STUDIES ON DEXTRANSUCRASE PRODUCTION BY *STREPTOCOCCUS SANGUIS* 804, NCTC 10904

An examination of dextransucrase production patterns at various pH values and the dextrans elaborated.

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The author (née Ayres) graduated from the University of Strathclyde with an Honours degree in Applied Microbiology in 1970. The results presented in this thesis were obtained between January, 1971, and September, 1974. The work has neither been submitted in any other thesis nor submitted for any other degree.

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CONTENTS.

		PAGE.
ACKNOWLEDGMENTS		ii
CONTENTS		iii
SUMMARY		xiii
1.	INTRODUCTION.	1
1.1	DENTAL CARIES.	1
1.2	THE DENTAL PLAQUE.	2
1.2.1	DEFINITION.	2
1.2.2	THE ROLE OF ADSORPTION AND PRECIPITATION OF SALIVARY COMPONENTS IN THE FORMATION OF THE ACQUIRED PELLICLE AND DENTAL PLAQUE.	2
	(i) THE ACQUIRED PELLICLE.	2
	(ii) ADSORPTION OF SALIVARY PROTEINS.	3
	(iii) PRECIPITATION OF SALIVARY PROTEINS.	3
1.2.3	THE ROLE OF BACTERIA IN PELLICLE AND PLAQUE FORMATION.	4
	(i) THE INCORPORATION OF BACTERIA.	4
	(ii) EXTRACELLULAR POLYSACCHARIDE PRODUCTION.	5
1.2.4	THE ROLE OF DEXTRANSUCRASE, LEVANSUCRASE AND THEIR PRODUCTS IN PLAQUE FORMATION AND DEVELOPMENT.	10
	(i) ADSORPTION.	10
	(ii) 'STICKINESS' OF DEXTRAN.	12
	(iii) THE MEASUREMENT OF PLAQUE-FORMING ABILITY OF DEXTRAN-PRODUCING BACTERIA.	13

1.2.5 THE ROLE OF AGGREGATION IN PLAQUE FORMATION AND DEVELOPMENT. 14

(i) AGGREGATION BY DEXTRAN. 14

(ii) OTHER AGGREGATING FACTORS. 15

(iii) AGGREGATION IN PLAQUE FORMATION. 15

1.3 DEXTRAN. 16

1.3.1 DEFINITION. 16

1.3.2 OCCURRENCE. 18

1.3.3 CHARACTERISATION OF PLAQUE DEXTRANS. 19

(i) PREPARATION OF DEXTRAN SAMPLES. 19

(ii) EXTRACTION OF DEXTRAN. 22

1.3.4 STRUCTURAL EXAMINATION OF DEXTRANS. 22

(i) PERIODATE OXIDATION. 23

(ii) SMITH DEGRADATION. 26

(iii) METHYLATION ANALYSIS. 26

(iv) ENZYMIC DEGRADATION OF DEXTRANS. 27

(v) INFRA-RED SPECTROSCOPY. 28

1.3.5 PHYSICAL PROPERTIES OF DEXTRAN. 30

1.3.6 PHYSICAL APPEARANCE OF DEXTRAN. 32

1.4 DEXTRANSUCRASE. 33

1.4.1 MODE OF ACTION. 33

(i) CHAIN ELONGATION. 34

(ii) ENZYME REQUIREMENTS. 34

(iii) INDUCTION. 34

(iv) INHIBITION. 36

1.4.2 OPTIMUM CONDITIONS OF DEXTRAN PRODUCTION. 36

(i) pH. 36

(ii) TEMPERATURE. 37

(iii) SUCROSE CONCENTRATION. 37

1.4.3 ENZYME PRODUCTION PATTERNS. 37

1.4.4 PURIFICATION OF DEXTRANSUCRASE. 38

(i) PRECIPITATION WITH $(NH_4)_2SO_4$ OR ACETONE. 39

(ii) ADSORPTION ONTO HYDROXYLAPATITE. 40

(iii) ISOELECTRIC FOCUSING. 40

(iv) CHROMATOGRAPHY ON DEXTRAN GELS. 41

(v) CHROMATOGRAPHY ON DEAE CELLULOSE. 41

(vi) SODIUM DODECYL SULPHATE GEL ELECTRO-PHORESIS. 42

1.4.5 THE ACTIVE SITE OF DEXTRANSUCRASE. 42

1.5 PLAQUE ECOLOGY. 42

1.5.1 ACID PRODUCTION. 44

1.5.2 LIMITATION OF GROWTH. 46

1.6 CONCLUSIONS. 48

1.7 AIMS OF THIS STUDY. 49

2. METHODS. 51

2.1 MATERIALS. 51

2.2 MICRO-ORGANISMS. 51

2.2.1 STREPTOCOCCUS SANGUIS. 51

2.2.2 STREPTOCOCCUS MUTANS. 51

2.3 MAINTENANCE MEDIUM. 52

2.4 CULTURE MEDIA. 52

2.4.1 CARLSSON'S DIFFUSATE MEDIUM. 52

2.4.2 NUTRIENT BROTH. 52

2.5 GROWTH OF S. SANGUIS. 52

2.5.1 RATE OF GROWTH AND pH CHANGE OF S. SANGUIS. 53

2.5.2 DEXTRANSUCRASE PRODUCTION BY S. SANGUIS. 53

2.6 ELUCIDATION OF OPTIMUM CONDITIONS FOR DEXTRANSUCRASE PRODUCTION. 53

2.6.1 THE EFFECT OF INOCULUM SIZE. 53

2.6.2 THE EFFECT OF INOCULUM AGE. 53

2.6.3 THE EFFECT OF pH CONTROL. 53

2.7 TREATMENT OF CULTURE SAMPLES. 56

2.8 BATCH CULTURE OF S. SANGUIS FOR DEXTRANSUCRASE PRODUCTION. 57

2.9 PREPARATION OF GLUCANS. 57

2.9.1 MUTAN 2 57

2.9.2 S GLUCAN. 59

2.9.3 DEXTRANS I, II AND III. 59

2.10 CHARACTERISATION OF DEXTRAN - CONTAINING PRECIPITATES. 59

2.10.1 GAS LIQUID CHROMATOGRAPHY. 59

2.10.2 HYDROLYSIS OF GLUCANS. 60

2.10.3 SPECIFIC AGGLUTINATION TECHNIQUE. 60

2.11 STRUCTURAL EXAMINATION OF DEXTRANS. 61

2.11.1 INFRA-RED SPECTROSCOPY. 61

2.11.2 ENZYMIC DEGRADATION. 61

2.11.3 PERIODATE OXIDATION. 61

2.12 PARTIAL PURIFICATION OF DEXTRANSUCRASE. 62

2.12.1 HYDROXYLAPATITE CHROMATOGRAPHY. 62

(i) BATCH-WISE ELUTION. 62

(ii) COLUMN CHROMATOGRAPHY. 62

2.12.2 ULTRAFILTRATION AND CONCENTRATION. 63

2.13 DIALYSIS AND CONCENTRATION OF MATERIAL. 63

2.13.1 DIALYSIS. 63

2.13.2 ULTRAFILTRATION AND CONCENTRATION. 63

(i) HOLLOW FIBRE DEVICE. 63

(ii) ULTRAFILTRATION CELL. 64

2.14 PROPERTIES OF DEXTRANSUCRASE. 64

2.14.1 OPTIMUM pH. 64

2.14.2 STABILITY. 64

2.15 ASSAYS. 65

2.15.1 CELL GROWTH. 65

2.15.2 DEXTRANSUCRASE. 65

(i) CARLSSON'S METHOD (1969). 65

(ii) CYBULSKA AND PAKULA'S METHOD (1963). 65

2.15.3 REDUCING SUGAR. 66

2.15.4 TOTAL CARBOHYDRATE. 66

2.15.5 GLUCOSE. 66

2.15.6 PROTEIN. 66

(i) LOWRY METHOD. 66

(ii) U.V. ABSORBANCE. 66

2.15.7 DEXTRANASE. 67

2.15.8 PERIODATE. 67

3. RESULTS. 68

3.1 GROWTH OF S.SANGUIS. 68

3.1.1 RATE OF GROWTH AND pH CHANGE OF S.SANGUIS CULTURES. 68

(i) THE TURBIDITY OF THE CULTURE IN RELATION TO THE DRY WEIGHT. 68

(ii) RATE OF GROWTH OF THE CULTURE. 68

(iii) pH CHANGE OF THE CULTURE. 68

3.1.2	DEXTRANSUCRASE PRODUCTION BY *S. SANGUIS*.	71
	(i) CHOICE OF DEXTRANSUCRASE ASSAY.	71
	(ii) THE USE OF ANTIBIOTICS IN CYBULSKA AND PAKULA'S MODIFIED DEXTRANSUCRASE ASSAY.	71
	(iii) DEXTRANSUCRASE PRODUCTION PATTERNS.	75
3.1.3	CONCLUSIONS.	75
3.2	DEVELOPMENT OF OPTIMUM CONDITIONS FOR DEXTRANSUCRASE PRODUCTION.	77
3.2.1	THE EFFECT OF INOCULUM SIZE.	77
3.2.2	THE EFFECT OF INOCULUM AGE.	80
3.2.3	THE EFFECT OF pH CONTROL.	80
3.2.4	THE EFFECT OF CONTAMINANTS.	87
3.2.5	CONCLUSIONS.	92
3.3	DEXTRANSUCRASE PRODUCTION PATTERNS.	94
3.3.1	CONCLUSIONS.	94
3.4	CHARACTERISATION OF THE PRODUCTS OF THE ACTION OF DEXTRANSUCRASE ON SUCROSE.	95
3.4.1	GAS-LIQUID CHROMATOGRAPHY.	95
3.4.2	ANALYSIS OF ACIDIC HYDROLYSATES.	95
3.4.3	SPECIFIC AGGREGATION OF *S. MUTANS*.	100
	(i) ELECTRON MICROSCOPY.	100
3.4.4	CONCLUSIONS.	100
3.5	STRUCTURAL EXAMINATION OF DEXTRANS.	103
3.5.1	INFRA-RED SPECTROSCOPY.	103
3.5.2	ENZYMIC DEGRADATION.	109
3.5.3	PERIODATE OXIDATION.	109
3.5.4	CONCLUSIONS.	113

3.6	PARTIAL PURIFICATION OF DEXTRANSUCRASE.	114
	3.6.1 HYDROXYLAPATITE CHROMATOGRAPHY.	114
	(i) BATCH-WISE ELUTION.	114
	(ii) COLUMN CHROMATOGRAPHY.	114
	3.6.2 CONCLUSIONS.	116
3.7	PROPERTIES OF DEXTRANSUCRASE.	116
	3.7.1 OPTIMUM pH.	116
	3.7.2 STABILITY.	120
	(i) STORAGE.	120
	(ii) HANDLING.	120
	3.7.3 CONCLUSIONS.	120
4.	DISCUSSION AND CONCLUSIONS.	124
5.	REFERENCES	138

LIST OF FIGURES

		PAGE
1.1	DIAGRAMMATIC REPRESENTATION OF THE STRUCTURE OF DEXTRAN.	6
1.2	DIAGRAMMATIC REPRESENTATION OF THE STRUCTURE OF LEVAN.	9
1.3	DIAGRAMMATIC REPRESENTATION OF THE STRUCTURE OF MUTAN.	17
2.1	FERMENTER FLASK ASSEMBLY FOR GROWTH OF S. SANGUIS UNDER CONDITIONS OF CONTROLLED pH.	54
2.2	FERMENTATION CELL FOR PRODUCTION OF DEXTRANSUCRASE FROM S. SANGUIS AT pH 7·1 $\pm$ 0·1.	58
3.1	THE RELATIONSHIP BETWEEN EXTINCTION AT 540 nm AND THE DRY WEIGHT OF THE CELLS/ml IN S. SANGUIS CULTURE SAMPLES.	69
3.2	GROWTH OF AND DEXTRANSUCRASE PRODUCTION BY S. SANGUIS.	70
3.3	THE TIME COURSE FOR DEXTRANSUCRASE ASSAY (1) AS DESCRIBED BY CARLSSON (1969).	72
3.4	THE TIME COURSE FOR DEXTRANSUCRASE ASSAY (2) AS DESCRIBED BY CARLSSON (1969).	73
3.5	THE TIME COURSE FOR A MODIFIED METHOD OF THE DEXTRANSUCRASE ASSAY DESCRIBED BY CYBULSKA AND PAKULA (1963).	74
3.6	DEXTRANASE ASSAY ON PENICILLIN/STREPTOMYCIN ANTIBIOTIC MIXTURE.	76
3.7	EFFECT OF INOCULUM SIZE AND AGE OF INOCULUM CULTURE ON GROWTH OF S. SANGUIS.	78
3.8	THE EFFECT OF INOCULUM SIZE ON DEXTRANSUCRASE PRODUCTION BY S. SANGUIS.	79
3.9	THE EFFECT OF INOCULUM CULTURE AGE ON DEXTRANSUCRASE PRODUCTION BY S. SANGUIS.	81
3.10	DEXTRANSUCRASE PRODUCTION BY S. SANGUIS AT pH 6·1 $\pm$ 0·1.	82
3.11	DEXTRANSUCRASE PRODUCTION BY S. SANGUIS AT pH 6·6 $\pm$ 0·1.	83

3.12	DEXTRANSUCRASE PRODUCTION BY S.SANGUIS AT pH 7·1 ± 0·1.	84
3.13	DEXTRANSUCRASE PRODUCTION BY S.SANGUIS AT pH 7·6 ± 0·1.	85
3.14	DEXTRANSUCRASE PRODUCTION BY S.SANGUIS AT pH 8·1 ± 0·1.	86
3.15	THE EFFECT OF pH CONTROL ON DEXTRANSUCRASE PRODUCTION AT pH 6, 7 AND 8.	88
3.16	THE EFFECT OF pH CONTROL ON DEXTRANSUCRASE PRODUCTION AT pH 6·5, 7 AND 7·5.	89
3.17	THE EFFECT OF CONTAMINATING BACTERIA ON THE PRODUCTION OF DEXTRANSUCRASE BY S.SANGUIS AT pH 6·6 ± 0·1.	90
3.18	THE EFFECT OF CONTAMINATING BACTERIA ON THE PRODUCTION OF DEXTRANSUCRASE BY S.SANGUIS AT pH 7·1 ± 0·1.	91
3.19	THE EFFECT OF A 2·5% INOCULUM OF A 16h CULTURE OF S.SANGUIS ON SUBSEQUENT GROWTH AND DEXTRANSUCRASE PRODUCTION.	93
3.20	G.L.C. OF PRODUCTS OF ACTION OF DEXTRANSUCRASE ON SUCROSE.	97
3.21	INFRA-RED SPECTRUM OBTAINED FOR DEXTRAN T2000.	104
3.22	INFRA-RED SPECTRA OBTAINED FOR GLUCANS IN THE REGION 600 - 1000 cm^{-1} (WAVENUMBER).	106
3.23	INFRA-RED SPECTRA OF GLUCANS.	108
3.24	ENZYMIC DEGRADATION OF GLUCANS BY MUTANASE.	110
3.25	ENZYMIC DEGRADATION OF GLUCANS BY DEXTRANASE.	111
3.26	PERIODATE CONSUMPTION BY GLUCANS.	112
3.27	HYDROXYLAPATITE CHROMATOGRAPHY OF DEXTRANSUCRASE II.	117
3.28	THE EFFECT OF pH ON DEXTRANSUCRASE ACTIVITY.	119
3.29	THE EFFECT OF STORAGE AT 4°C ON DEXTRANSUCRASE ACTIVITY.	121
3.30	THE EFFECT OF FREEZING AND THAWING ON DEXTRANSUCRASE ACTIVITY.	122

LIST OF TABLES

		PAGE.
1.1	DIAGRAMMATIC REPRESENTATION OF THE CARBOHYDRATE CONTENT OF DENTAL PLAQUE. (HOTZ ET AL, 1972).	20
1.2	PERIODATE OXIDATION PRODUCTS OF α-(1→6)-, α-(1→4)- AND α-(1→3)-LINKED GLUCANS.	25
1.3	THE STRUCTURAL EXAMINATION OF DEXTRAN PREPARATIONS.	35
1.4	PURIFICATION OF DEXTRANSUCRASES OF PURE CULTURES OF PLAQUE BACTERIA.	43
3.1	ACIDIC HYDROLYSIS OF GLUCANS.	99
3.2	BATCH-WISE ELUTION OF DEXTRANSUCRASE FROM HYDROXYLAPATITE.	115
3.3	PURIFICATION OF DEXTRANSUCRASE II BY HYDROXYLAPATITE COLUMN CHROMATOGRAPHY.	118

PLATES

3.1 3.4 ELECTRON MICROGRAPHS OF AGGREGATED CELLS OF S. MUTANS. 102

SUMMARY

Streptococcus sanguis 804 is a microaerophilic organism which was originally isolated from human dental plaque (Carlsson, 1965). It is one of the first organisms to colonise the tooth surface during dental plaque formation and is the predominant aerobic streptococcus in young plaque (Carlsson, 1965). It produces the constitutive, extracellular enzyme, α-(1→6)glucan : D-fructose 2-glucosyltransferase (EC 2.4.1.5), commonly known as dextransucrase, which polymerises the glucose moiety of sucrose to form dextran, with the release of fructose.

The dextran it produces is believed to contribute to dental plaque formation and the initiation of dental caries.

This study examined dextransucrase production by S.sanguis at different pH values in order to elucidate optimal conditions for its production. The optimum pH was found to be in the region of 7·1 $\pm$ 0·1 but, it emerged, that at all pH values studied the enzyme was produced in three, distinct, reproducible phases. Dextran was prepared from enzyme harvested at each of the three phases at pH 7·1 $\pm$ 0·1 and, after characterisation of the substances as dextran by gas-liquid chromatography, acidic hydrolysis and a specific aggregation technique, they were examined for structural differences by infra-red spectroscopy, periodate oxidation and enzymic degradation.

The results suggest that the three preparations differ in structure. Crude enzyme prepared from culture supernatant at the second or third peak of enzyme activity was partially purified by hydroxylapatite chromatography. Enzyme from the second peak had a pH optimum in the region 5·2 - 7·5.

It is suggested that there may be more than one enzyme involved in dextran production by S.sanguis 804.

1. INTRODUCTION

1.1 DENTAL CARIES.

In 1890, W.D. Miller stated that he believed dental caries to be initiated by the acids produced by bacterial fermentation of foodstuffs, particularly carbohydrates, but he was unable to explain why decay was localised to specific areas of the tooth instead of a more universal attack or why the acid was not rapidly rendered harmless by dilution with saliva.

In 1897, G.V. Black advanced his theory to explain the localised decay process. He believed that a "gelatin-like substance of a gluey consistency" produced by oral bacteria protected them from removal from the smooth tooth surface and that the gel trapped acids within it, holding them against the tooth surface and separating them from the saliva. These acid accumulations in close proximity to the tooth surface could then initiate the localised decay process.

Black also believed that sucrose was necessary to produce such a gel and he felt that the ability to form this substance was the factor controlling the initiation of caries. He termed the accumulation of bacteria and gelatin-like material 'gelatinous microbic plaque'.

His theory was finally accepted and has remained essentially unchanged to this day.

The definition of caries, according to Scopp (1973) is that "tooth decay is a bacterial infection that develops when the mineral components of enamel dissolve in acids formed by bacterial accumulation on the tooth surface. The interactionsbetween specific bacteria, diet and tooth susceptibility combine to produce caries".

1.2 THE DENTAL PLAQUE.

1.2.1 Definition. Dental plaque is extremely difficult to define due to its heterogeneous and dynamic nature (Critchley and Saxton, 1970). It varies from one individual to the next and even within the same individual with diet (Bowen and Critchley, 1970), age of the plaque (Carlsson, 1965, Gibbons and van Houte, 1973), location in the mouth (Bibby, 1938, and Critchley, 1969), degree of oral hygiene (Pickerill, 1915) and even with the site on a particular tooth (Stephan, 1948, Wilcox and Everett, 1963).

On exposure of a thoroughly cleaned tooth surface to oral fluids, an acellular, bacteria-free layer 1 -15 μm thick is formed within minutes (Leach, 1967, Schroeder and De Boever, 1970). This is known as the acquired pellicle or pellicle.

Further exposure of the tooth to mixed saliva in the oral cavity results in the invasion of the pellicle by bacteria which surround themselves with a matrix distinctly different from the pellicle (Schroeder and De Boever, 1970). This accumulation of bacteria and matrix is known as dental plaque.

1.2.2 The role of adsorption and precipitation of salivary components in the formation of the acquired pellicle and dental plaque.

(i) The acquired pellicle. The composition of pellicle, unlike plaque, appears to be fairly constant and does not vary according to its site of origin (Sönju and Rölla, 1972, Belcourt et al, 1974). It is believed to consist primarily of adsorbed salivary proteins (Section 1.2.2.ii) as its amino acid composition is qualitatively very similar to that of salivary glycoproteins (Leach, 1967). However, the ratio of amino acids in pellicle is not the same as that in salivary glycoprotein. There appears to be a selective adsorption of salivary components (Ericson, 1967) --

particularly acidic amino acids -- to the tooth surface (Section 1.2.2.ii) and this process followed by bacterial degradation of the pellicle (Ericson, 1967) may alter the ratio of constituents from that found in saliva. Further chemical changes may occur as the pellicle rapidly transforms to a tough, protective layer which is highly resistant to acid attack (Meckel, 1968). Some blood group substance activity has been detected in pellicle (Sönju and Rölla, 1972) as have bacterial cell wall constituents (Meckel, 1968), suggesting that gingival fluid and bacterial cell walls might also contribute to its formation.

(ii) Adsorption of salivary proteins. The inorganic constituent of dental enamel which forms 95% by weight is predominantly hydroxylapatite, (Napper and Smythe, 1966) a substance which selectively adsorbs certain proteins (Bernardi et al,1972). Salivary glycoproteins, especially those containing sialic acid residues bind strongly to hydroxylapatite (Sönju and Rölla, 1972, Ericson, 1967). Other proteins of oral fluids have also been shown to be adsorbed (Örstavik and Kraus, 1974, Rölla and Mathieson, 1970, Schroeder and De Boever, 1970). As explained in Section 1.2.2.i, this adsorption occurs in pellicle formation, but it continues throughout the build-up of dental plaque. In fact, evidence suggests that adsorption of proteins by hydroxylapatite is enhanced by the presence of pellicle (Meckel, 1968, Rölla and Mathieson, 1970). Certain native proteins, including enzymes, may be reversibly bound to denatured proteins of pellicle and plaque, allowing their desorption and diffusion into plaque (Örstavik and Kraus, 1974) -- Section 1.2.3.

(iii) Precipitation of salivary proteins. Salivary proteins may be precipitated directly onto the enamel surface or adsorbed after precipitation has occurred. There are several theories concerning the mode of precipitation. These include the enzymic breakdown of glycoproteins by

bacterial enzymes such as neuraminidase (Fukui et al, 1971), which raises the isoelectric point of the protein, causing its precipitation, the spontaneous precipitation of saliva frequently observed when it is collected, acidification of the saliva by bacterial action, the presence of calcium ions which can enhance protein precipitation and the effect of the ionic strength of saliva. These theories have been discussed in several reviews (Jenkins 1968, Jenkins 1972, Gibbons and Van Houte, 1973, Kleinberg 1973, Leach 1970).

Much of the research conducted into pellicle and plaque formation has been carried out on samples collected on prosthetic devices employing strips of Mylar, celloidin, glass and other synthetic materials in the place of enamel (Egelberg 1970, Bruckner 1948, Naylor et al, 1970, Ennever et al 1948, Schroeder and De Boever 1970). The validity of these experiments is, therefore, questionable as only passive deposition of materials is possible. Adsorption of protein at the charged enamel surface cannot be observed in these experiments and such differences at this early stage of plaque formation could have far-reaching effects upon subsequent stages of plaque development. A great deal of the work on salivary glycoproteins has been conducted on animal saliva as this is readily obtainable from abattoirs, but its relevance to human saliva is not known (Leach, 1967).

1.2.3 The role of bacteria in pellicle and plaque formation.

(i) The incorporation of bacteria. Clumps of approximately 50-200 bacteria, rather than individual cells, appear to attach to the enamel surface initially. This would be expected to lead to a more rapid colonisation of the tooth than would the attachment of single cells (Critchley and Saxton, 1970). The cells may be aggregated by factors present in the oral fluid prior to their adsorption to the tooth surface - Section 1.2.4 (Gibbons and Van Houte, 1973).

The bacterial flora of young plaque is not the same as that of saliva and it appears that incorporation of bacteria is not random but a selective adsorbtion (Gibbons and Van Houte, 1973) of cell wall components or polysaccharide capsules to hydroxylapatite (Clark et al, 1974, Meckel, 1968).

(ii) Extracellular polysaccharide production. Attempts to identify the matrix material of dental plaque met with little success initially. Confusion arose when attempts were made to show that the matrix material was of salivary origin by using a technique which stained glycoprotein. The material took up the stain but it was not realised that the technique was not specific for glycoproteins - it merely stained the sugars in the glycoprotein molecule and would, therefore, give a positive reaction with any material containing sugars (Leach, 1967). The matrix takes up carbohydrate stains well (Barkin, 1970, Critchley and Saxton, 1970), and is now known to be primarily carbohydrate in nature. Characterisation of this carbohydrate material has proved to be extremely difficult due to problems of extraction of the material in sufficient quantities for chemical analysis. These difficulties will be discussed in Section 1.3.3.ii.

The carbohydrate is now believed to be a mixture of dextrans, levans and heteropolysaccharides (Guggenheim, 1970).

The dextran was assumed to have the same structure as that produced by Leuconostoc mesenteroides (Fig. 1.1.). This is a branched polymer of glucose units with α-(1→6)-linkages in the main chain and α-(1→4)-, α-(1→2)- or α-(1→3)- linked side branches (Jeanes et al, 1954). It is produced by an extracellular enzyme - α-(1→6)-glucan : D-fructose 2-glucosyltransferase (EC 2.4.1.5), commonly known as

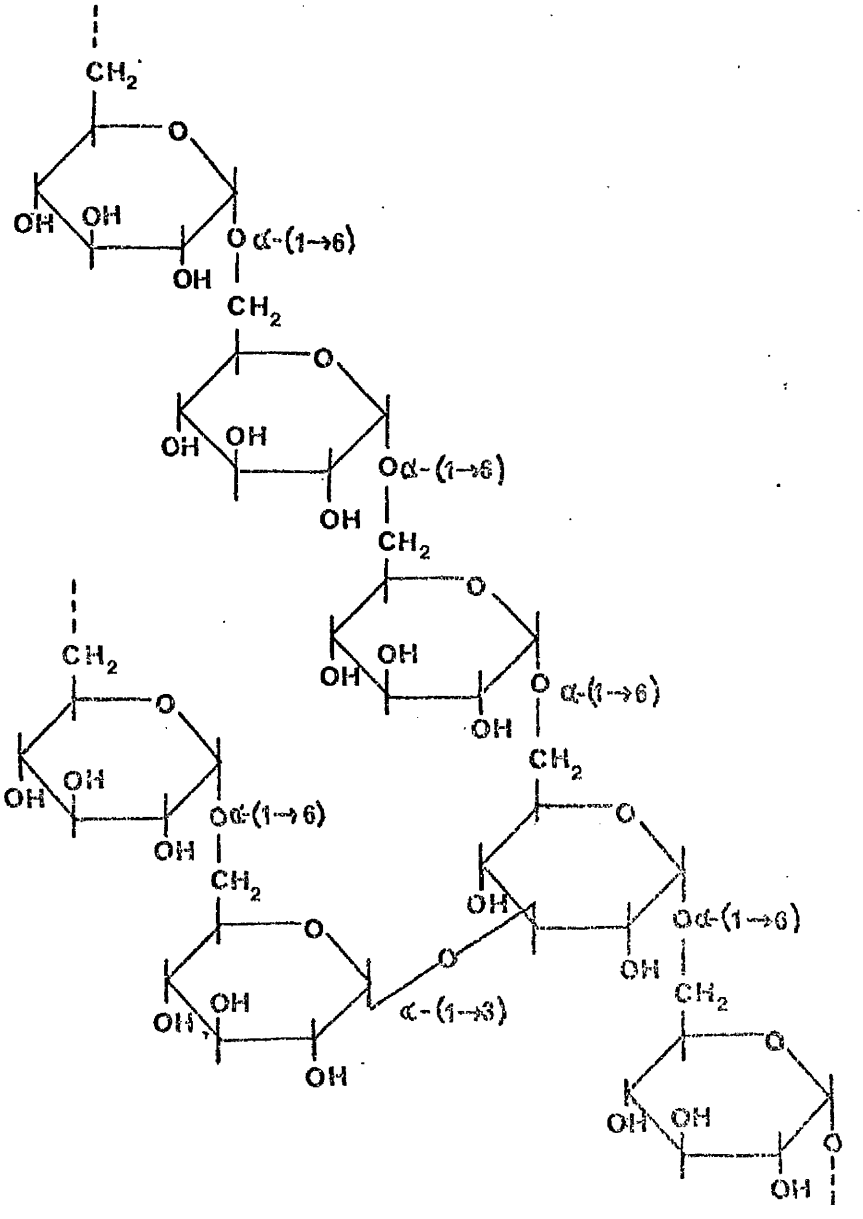


FIG.1.1 DIAGRAMMATIC REPRESENTATION OF THE STRUCTURE OF DEXTRAN.

dextransucrase, according to the following equation:-

$$(\text{Glucose})_n + y\,\text{Sucrose} \rightarrow (\text{Glucose})_{n+y} + y\,\text{Fructose}$$

Dextran Dextran

(See Section 1.4).

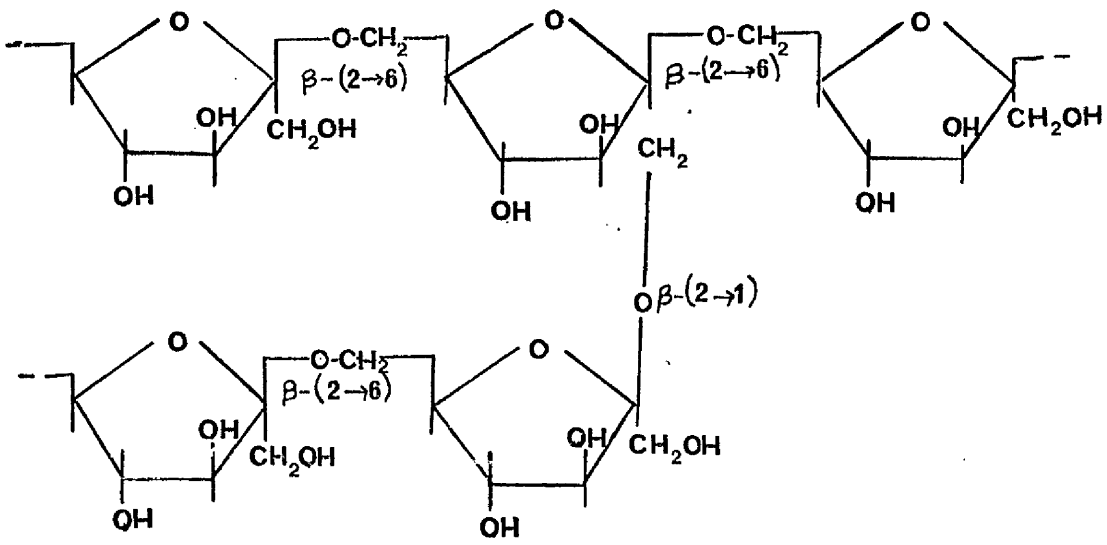
Levan is a polymer of fructose units linked by β- (2→6) - linkages with β- (2→1) - linked side branches (Fig.1.2). It is formed according to the following equation by the enzyme β-(2→6) - fructan : D - glucose - 6 - fructosyltransferase (EC 2.4.1.10), commonly known as levansucrase.

$$(\text{Fructose})_n + y\,\text{Sucrose} \rightarrow (\text{Fructose})_{n+y} + y\,\text{Glucose}$$

Levan Levan

Unlike dextran, levan is readily soluble in water and, as it only makes up 1 - 2% of the total dry weight of plaque (McDougall, 1964) due to its very rapid turnover rate (Gibbons and Banghart, 1967, Higuchi et al, 1970) it is not thought to be an important structural component of plaque. Dextran and levan are formed specifically from sucrose, approximately 15% of the sucrose being converted to polysaccharide by plaque bacteria, the rest being metabolised with release of acid (Tanzer et al, 1972, Baird et al, 1973).

FIG.1.2. DIAGRAMMATIC REPRESENTATION OF THE STRUCTURE OF LEVAN.


O
O-CH2
β-(2→6)
OH
CH2OH
CH2
β-(2→1)

Other polysaccharides have also been found in plaque, particularly when sucrose is missing from the diet (Guggenheim, 1970, Bowen and Cormick, 1970). They have been identified as heteroglycans, similar to those found in bacterial cell walls (Hotz et al, 1972, Guggenheim 1970, Dunnican and Seeley, 1966).

Dextran and levan are produced specifically from sucrose (Section 1.4.) but the heteroglycans may be produced from other sugars in addition to sucrose (Newbrun 1969, Guggenheim 1970). Studies on the effect of sugar mouthrinses on plaque have shown that in the presence of sucrose - the substrate necessary for dextran and levan production - a more voluminous plaque is formed than with other sugars (Carlsson and Egelberg, 1965) and that the polysaccharide matrix seen under the electron microscope increases after sucrose rinses (Saxton 1969). Many of the bacteria isolated from the mouth and throat will, in the presence of sucrose, produce mucoid colonies on solid medium and thick gels in liquid medium. These gels consist of extracellular polysaccharides, particularly dextran and levan, which are produced in copious amounts. The bacteria which produce them are commonly found in dental plaque but much less frequently in the oral fluids and in other areas of the mouth (Snyder et al, 1955). The implantation of these bacteria in the mouth is greatly facilitated by the presence of sucrose in the diet (Krasse, 1965) and the possibility that the ability to produce extracellular polysaccharide, particularly dextran and levan, may be a key factor in the selection of bacteria for plaque formation therefore merited further investigation (Section 1.2.4).

1.2.4 The role of dextransucrase, levansucrase and their products in plaque formation and development.

(i) Adsorption. The role of adsorption of proteins to hydroxylapatite has already been discussed (Section 1.2.2.ii) but dextrans, dextransucrase, levansucrase and dextran-coated cells have also been shown to bind strongly to

hydroxylapatite (Ericson, 1967, Clark et al, 1974, Carlsson, 1970, Chludzinski et al, 1974) particularly if it is protein coated (Rölla and Mathieson, 1970).

Most proteins bound to the enamel surface are believed to be in a denatured form (Jenkins, 1968) but work by Örstavik and Kraus (1974) on pellicle has shown that some proteins may be reversibly bound to pellicle in the native form, retaining their biological activity. This binding of, for example, an enzyme, to an insoluble matrix brings substrate and enzyme into close contact, allowing the products to diffuse away but not the enzyme. This is a very efficient system (Mosbach, 1972). If this is the case for dextransucrase, its adsorption to the enamel surface at an early stage of plaque formation could have additional significance, as explained below.

Dextransucrase has a very high affinity for dextran. The enzyme occurs extracellularly and can therefore be carried in the oral fluids to areas distant from the cell which produced it and where bacterial colonisation may not yet have occurred. It may then be adsorbed to pellicle in an active form and, in the presence of sucrose, produce dextran, distant from its site of origin. Alternatively, in the absence of sucrose, the enzyme's high affinity for dextran may lead to the incorporation of dextran molecules or dextran coated bacteria from the oral fluids, into the dextransucrase containing plaque, thus contributing to its accumulation. It is interesting to note in this connection that only a small proportion of the dextransucrase (approx. 10%) produced by oral bacteria is present within the cell, (Sharma et al, 1973) or upon its surface (McCabe and Smith, 1973, Gibbons and Spinell, 1974). Intracellular enzyme can be released upon rupture of the cell but the exact location of the enzyme within the cell has not yet been investigated.

Cell-bound dextransucrase may, in the absence of sucrose to form its own dextran, become bound to dextran already elaborated and present upon other cells or the pellicle. This adsorption of dextransucrase by dextran is much more specific than the adsorption of dextran or dextransucrase to hydroxylapatite which competes with other proteins, carbohydrates and bacterial cell wall components.

The interaction between dextran and dextransucrase may lead to the clumping together of cells and may be the basis of certain types of aggregation observed in oral bacteria (Section 1.2.5). For this phenomenon to occur some cells must produce dextransucrase at the cell surface where it is readily available (Gibbons and Spinell 1974). This has been shown to be the case for S. mutans, one of the species which dominates early plaque formation. There is also evidence to suggest that dextran may bind to specific sites upon bacterial cell walls which are distinct from the sites of dextransucrase activity (Gibbons and Spinell, 1974). These dextran-binding sites may be located on the dextransucrase molecule but distinct from the site of enzymic activity. Callaham and Heitz (1973) have demonstrated that such sites do occur, in close proximity to each other on the dextransucrase from S. sanguis 804.

(ii) Stickiness of dextran. One of the factors believed to be of importance in plaque formation is the 'stickiness' conferred upon the cells by the dextran coating produced in the presence of sucrose. Dextran chains produced by Leuconostoc mesenteroides are believed to adopt a helical arrangement, (Ebert and Schenk, 1968) stabilised by hydrogen bonds (Section 1.3.5) and this 'spring-like' structure conveys the property of 'stickiness' to dextran. These molecules, however, are only about 5% branched and the branches tend to be short (Ebert and Schenk, 1968). Native streptococcal dextran is a thick, gelatinous and

highly tenacious substance with a complex, branched structure. Presumably a helical arrangement would not be possible due to the highly branched nature of the molecule but bacterial cells, epithelial cells, food particles, etc., may become entrapped within the branches. It is streptococcal dextran which occurs in plaque and not the dextran produced by leuc.mesenteroides. Freeze-etching and electron microscopy studies on plaque have revealed the production of three different forms of dextran in progression. Initially globules of dextran appear at specific sites on the cell wall, possibly the sites of dextransucrase production, which rapidly enlarge and coalesce forming an amorphous mass around the cell. Spaces develop between the cell wall and the dextran which may then coalesce with the dextran of neighbouring cells. Strands of dextran then appear, linking neighbouring cells together and finally a fine, fibrillar network covers all the cells. Thus the plaque cells are held together by an intricate network of dextrans which is produced within minutes of exposure to sucrose (Saxton and Findlay, 1971). This elaboration of several structurally distinct dextrans has led workers to believe that there may be more than one dextransucrase, each one producing a dextran of a slightly different structure. It also seems likely that such a complex, highly branched molecule might be formed by more than one enzyme; there might be different enzymes for each linkage type or branching enzymes.

(iii) The measurement of plaque-forming ability of dextran-producing bacteria.

The ability of oral bacteria to form plaque has been measured by suspending sterile wires in sucrose supplemented broths of the test organism (Fitzgerald et al, 1968, McCabe et al,1967). The use of wires precludes the possibility of adsorption of the cells or dextran to hydroxylapatite. However, only dextransucrase producing organisms formed deposits on the wires and no deposit formed when dextranase was included in the incubation mixture (Fitzgerald et al, 1968). A direct relationship was shown between the

ability to form a plaque-like deposit and cariogenic potential. The latter is measured as the ability of the organism to become established in the mouths of gnotobiotic animals and to initiate caries (Guggenheim, 1968). Organisms which produce both dextran and levan do not form plaque-like deposits in the presence of dextranase, suggesting that levan does not play a significant role in plaque formation (Gibbons and Nygaard, 1968) but Parsons et al 1973 have implicated levan in plaque formation in mixed cultures. However, non-plaque producing bacteria were often capable of producing plaques containing both bacterial species when grown in mixed culture with another organism (Miller and Kleinmann, 1974, Parsons et al, 1973, Gibbons and Keyes, 1969).

1.2.5 The role of aggregation in plaque formation and development.

(i) Aggregation by dextran. The ability of dextran to 'agglutinate' cells of S.mutans has already been mentioned (Section 1.2.4). Gibbons and Fitzgerald (1969) used the term 'agglutination' to describe this phenomenon but as this implies the involvement of agglutinins, the term aggregation is preferable.

S.mutans produces dextransucrase at the cell surface and, upon addition of sucrose, the cells rapidly become coated with dextran, cohere and aggregation occurs. Addition of dextranase [α - (1→6) - glucan hydrolase] to the cells with the sucrose prevents dextran formation and the cells do not aggregate. Addition of any high molecular weight dextran (McCabe et al, 1974) including those produced by other bacteria, to S.mutans cells brings about rapid aggregation but other polysaccharides such as starch, inulin, levan, dextrin, agar, agarose or low molecular weight dextran have no effect (Gibbons and Fitzgerald, 1969). It is a very sensitive, highly specific phenomenon which can be used to detect the presence of dextran (Section 2) and as little as 3 or 4 molecules of high molecular weight dextran per cell will cause aggregation. The aggregated cells are not easily dispersed and remain stable over the pH

range 5 - 10 which covers most of the pH values recorded in the oral environment i.e., 4-5 - 8 (Stephan, 1948, De Boever and Muhlemann, 1969). They are dispersed, however, by cyclohexyl isocyanide and byurea, suggesting the involvement of hydrogen bonds. EDTA also causes partial dispersion. The cells can be re-aggregated by the addition of certain divalent cations but are dispersed again, in some cases by heating. The dispersion is reversible, the cells re-aggregating on cooling. It is thought that divalent cations form 'bridges' between negatively charged groups and that when the hydrogen bonds are broken, the cations are chelated by EDTA. Dextran is produced by many plaque bacteria and S.mutans is one of the most common bacteria in young plaque. The above mechanism is probably of great importance, therefore, in early plaque formation.

(ii) Other aggregating factors. Other factors capable of aggregating bacteria have been found but few have been identified. They include salivary components and bacterial products (Gibbons and Spinell, 1970, Williams and Gibbons, 1974, Chatterjee and Kleinberg, 1974, Gibbons and Van Houte, 1973). This could explain why mixed cultures of non-plaque producing bacteria acquire the ability to form plaque-like deposits (Section 1.2.4iii).

(iii) Aggregation in plaque formation. The full extent of the role of aggregation in plaque formation is not yet known but one group of workers alone found that of 62 plaque isolates, 28 were aggregated by saliva (Gibbons and Spinell, 1970).

Electron microscopy studies on plaque development have revealed that the initial colonisation of the tooth surface may be aggregated cells (Section 1.2.3i).

Groups of coccoidal organisms have been seen clustered around a central filamentous bacteria to give a 'corn on the cob' arrangement (Gibbons and Van Houte, 1973). The cells are embedded in extracellular polysaccharide

and it is thought that aggregation may have occurred or the coccoidal organisms may be metabolising the polysaccharide elaborated by a filamentous organism (Gibbons and Van Houte, 1973, Critchley and Saxton, 1970).

Plaque, then, is a constantly changing material with metabolic products diffusing out of it and substances from food, saliva and bacterial metabolism accumulating upon its surface. The bacteria surround themselves in a polysaccharide matrix forming their own micro-environment in which to live. The formation of dental plaque is a prerequisite to the initiation of caries and it has long been thought that plaque may trap metabolic acids produced by the bacteria, in close proximity to the tooth surface thus initiating caries.

Sucrose is known to be highly cariogenic. In order to understand the role of dextran in plaque ecology and the carious process, it is necessary to obtain some information about its structure and physical properties.

1.3 DEXTRAN.

1.3.1 Definition. The term dextran is applied to homopolysaccharides of (1→6)- linked α-glucopyranose monomers which may exist as straight chains or possess α-(1→3) -, α-(1→2) or α-(1→4)- linked side branches. The degree of branching is usually less than 20% and the side chains are frequently short (Heyn, 1974).

Recent work on dextrans from oral bacteria has revealed that many have a very high α-(1→3)- linkage content and α-(1→3) linkages may be the predominant linkage type (Table 1.3). Strictly speaking, the term dextran is not applicable to such a structure and the term 'mutan' has been proposed (Fig.1.3.). However, in the absence of a satisfactory rule for the nomenclature of such polyglucans and the absence of information about the structure of many of them, the term dextran will continue to be used to avoid confusion.

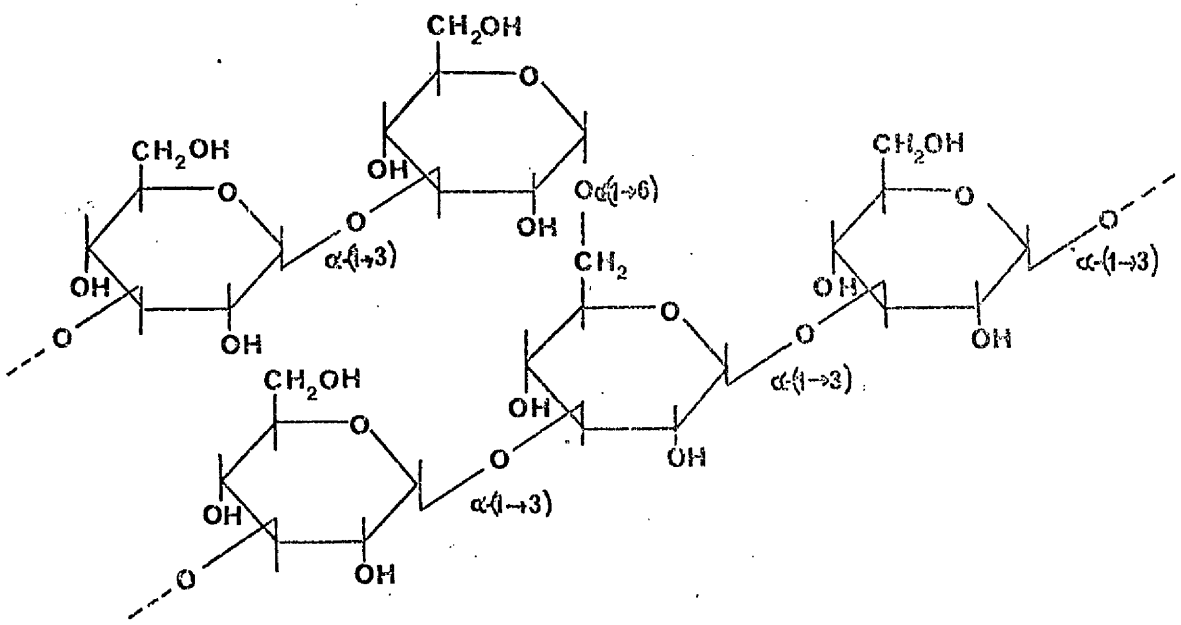


FIG.1.3 DIAGRAMMATIC REPRESENTATION OF THE STRUCTURE OF MUTAN.

1.3.2 Occurrence. Most of the early work on dextrans was performed on the dextran produced by Leuc.mesentercides, a bacterium associated with spoiled vegetables and milk products and slimy sugar solutions (Topley and Wilson, 1966). The slime or 'ropiness' attributed to this organism was due to the production of dextran from sucrose.

The dextran was found to be useful in medicine as a blood plasma extender to treat shock and this stimulated a great deal of interest in it during the war years. However, dextrans produced by other organisms were largely ignored except as a taxonomic aid in the identification of bacterial isolates from patients suffering from bacterial endocarditis (Niven et al, 1946, Mehre and Neill, 1946). Most of these isolates could be placed in one species which was named Streptococcus sanguis. Dextrans produced by bacteria of the mouth and pharynx were long regarded as unimportant due to their "relative rarity" (Snyder et al, 1955) but the discovery of the dextran-producers, S.sanguis and S.mutans in dental plaque (Carlsson, 1965) and the theory that the ability to produce dextran contributed in some way to the selection and incorporation of bacteria from the oral environment into dental plaque (Gibbons and Nygaard, 1968, Krasse, 1965, Gibbons, 1968b) once more focussed attention upon them.

1.3.3. Characterisation of plaque dextrans. This has already been discussed briefly in Section 1.2.3ii. Examination of plaque samples before and after rinsing with dextranase showed a reduction in volume of the polysaccharide material, suggesting the presence of dextran which was hydrolysed by the enzyme. However, there are problems associated with the use of this enzyme which have led to an erroneous impression about the amount and structure of the dextran present (Section 1.3.4iv).

Another method frequently used to identify dextran was the preparation of antisera to it and incubation of the antiserum with the test material. The production of a precipitate was taken to indicate the presence of dextran

(Gibbons and Banghart, 1967). However dextrans have been shown to cross-react with antisera to other polysaccharides including heteropolysaccharides such as those produced by other plaque bacteria and the test cannot be assumed to be specific (Hammond, 1969, Dunnican and Seeley, 1965).

It was, for a long time, assumed that only one dextran was present but it is unlikely that different bacteria or different strains of a particular organism would all produce exactly the same dextran. Even slight differences in structure could be expected to cause differences in properties such as solubility. Conditions within plaque are constantly changing and this must reflect on the dextrans produced (Jeanes et al, 1954).

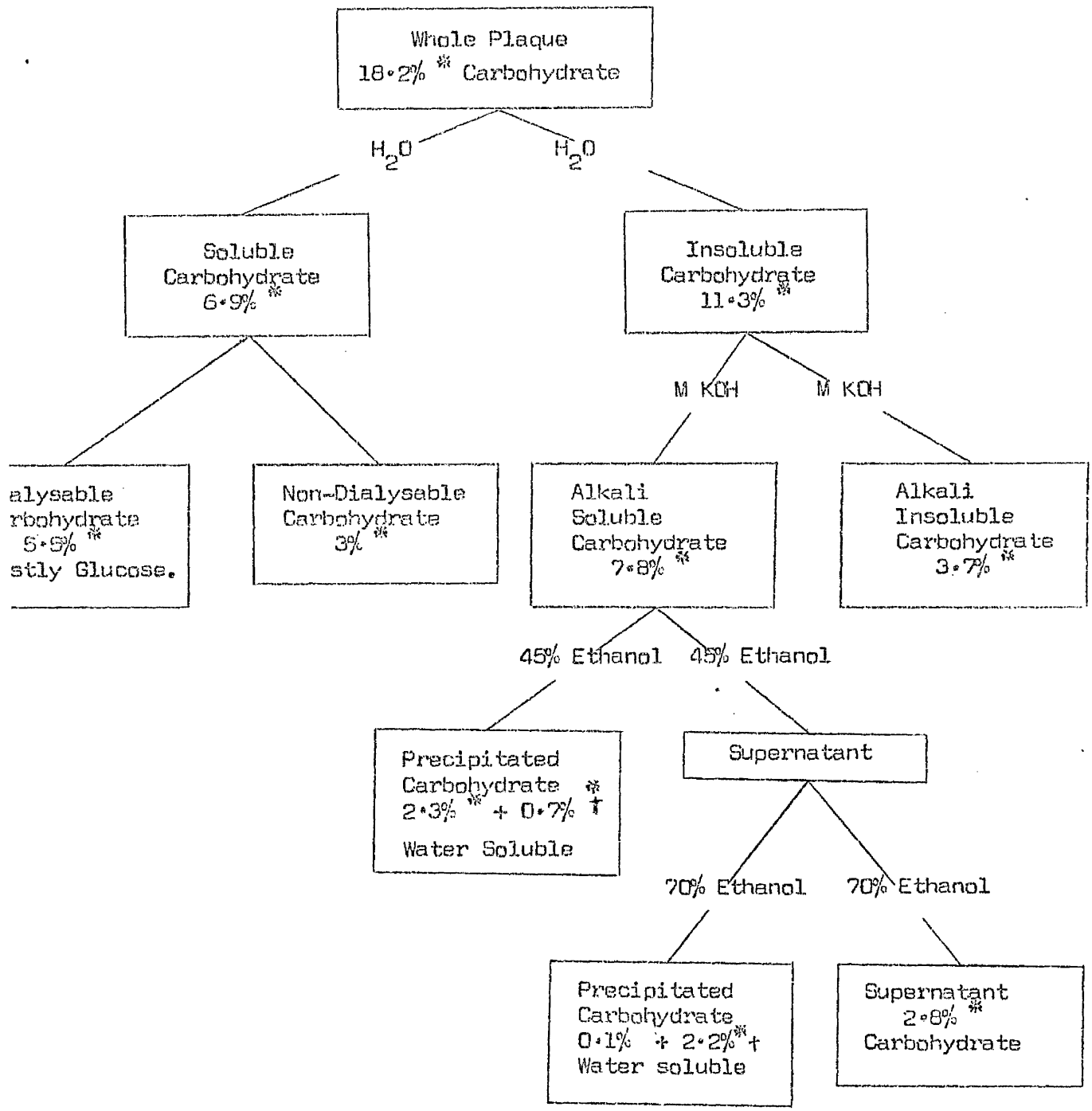
It is now recognised that dextrans produced by oral bacteria can be fractionated in accordance with their relative solubilities in water and increasing concentrations of alkali (Baird et al, 1973).

Acidic hydrolyses of dextran samples extracted from plaque or from pure cultures of plaque bacteria have shown it to be a homopolymer of glucose and the samples are usually 80-100% pure carbohydrate (Gold et al, 1973, Long, 1971, Newbrun, 1972). Most dextrans contain 5-10% moisture (Critchley, 1969, Wood, 1967, Newbrun, 1972) but it is not always clear if workers adjust data to allow for this. Table 1.1. summarises the way in which the carbohydrate content of plaque can be fractionated (Hotz et al, 1972).

(i) Preparation of dextran samples. A great deal of the present knowledge about dextrans produced by plaque bacteria has been gained through work on pure cultures of dextran-producing plaque bacteria. It is assumed that these dextrans are produced in dental plaque but it is realised that, as the conditions prevalent in plaque, cannot be reproduced exactly in the laboratory, the results must be interpreted with caution.

The dextran-producing bacteria studied have largely been the so-called cariogenic strains (Section 1.2.4iii). However, bacteria which are not

TABLE 1.1 DIAGRAMMATIC REPRESENTATION OF THE CARBOHYDRATE CONTENT OF DENTAL PLAQUE (HOTZ et al, 1972).



* Percentages expressed as % of dry weight of plaque.

† Soluble carbohydrate released on precipitation. (Possibly low molecular weight sugars trapped within more complex carbohydrate structure).

cariogenic under these conditions might, in a suitable combination with other oral bacteria, become capable of initiating caries. Evidence which suggests that this might occur has been put forward by Krasse (1970) and Hadley (1924).

The dextran must first be produced from the organism selected for study. Conditions of production vary, almost from one research team to the next and it is not generally recognised that growth conditions are important and could have a profound effect upon the dextransucrase and hence the dextran produced (Ceska et al, 1972, Jeanes et al, 1954).

The growth medium also varies and is not usually examined for its suitability. For example, the presence of high molecular weight proteins in the medium which would be precipitated by the ethanol used to extract the dextran could cause problems of contamination (Cybulska and Pakula, 1963). It is also known that dextransucrase production is better in some media than others (Cybulska and Pakula, 1963) and the proportion of different dextransucrase can vary with the medium (Gibbons and Spinell, 1974). In some cases sucrose is added directly to the culture so that dextran is produced (Gold et al, 1973, Gibbons and Bonghart, 1967). This causes problems in harvesting the dextran as insoluble polysaccharide is lost with the bacterial cells when the culture is centrifuged (Hammond, 1969) or, in attempts to extract this insoluble material from the cells, cell wall polysaccharides may also be extracted to contaminate the dextran (Gibbons and Nygaard, 1968, Smith 1970-71). The dextran produced is also more likely to be contaminated with medium constituents. The poor extraction procedures often led to insoluble polysaccharide being lost and thus overlooked. The water soluble nature of the polysaccharides studied led to the conclusion that they could not play an important part in plaque production (Snyder et al, 1955).

Other workers cultured the cells in the presence of glucose and used the

crude culture supernatant as a source of dextransucrase (Baird et al, 1973). The dextran produced was more easily harvested and less seriously contaminated. Cultivation of the bacteria has also varied with respect to the use or absence of pH control (Baird et al, 1973, Cybulska and Pakula, 1963) which could affect the enzyme produced (Gibbons and Nygaard, 1968). Other variables in the production of dextrans are the degree of aeration, stirring and oxygenation and the stage at which the culture is harvested. Thus it is difficult to make valid comparisons between dextrans as conditions of production vary so widely.

(ii) Extraction of dextran. Conditions of extraction of dextran from the incubation mixture and from plaque also vary (Krembel et al, 1969). Insoluble dextrans may be centrifuged down initially and the supernatant treated with ethanol to precipitate the water-soluble dextrans. A final concentration of 50-90% ethanol is necessary to precipitate all the dextran (Manly and Kerrigan, 1972) but as the concentration of ethanol increases, levan, which is also produced by many dextran-producing bacteria, is precipitated (Manly and Kerrigan, 1972, Baird et al, 1973, Newbrun, 1972) and may contaminate dextran preparations. In some cases the bacterial cells are disrupted to release cell-bound enzyme (Gibbons and Keyes, 1969, Hammond, 1969, Gibbons and Nygaard, 1968) and this may produce a dextran which differs from that produced by extracellular enzyme (Gibbons and Nygaard, 1968).

The dextrans may be fractionated by extraction with water and increasing concentrations of KOH or NaOH (Baird et al, 1973, Gibbons and Nygaard, 1968, Gibbons and Banghart, 1967). The method of extraction may influence the molecular arrangement of the dextran and hence its chemical reactions, as shown by Wessels et al (1972) for an α-(1→3) - linked-glucan from the cell walls of Schizophyllum commune.

1.3.4 Structural examination of dextrans. In order to study the type of linkage present in dextran, periodate oxidation and methylation studies have been very useful (Sloan et al, 1964, Rankin and Jeanes, 1954).

Enzymic degradation has also been employed but is of limited use (Section 1.3.4.iv).

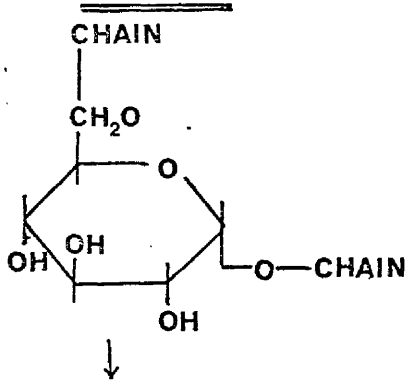
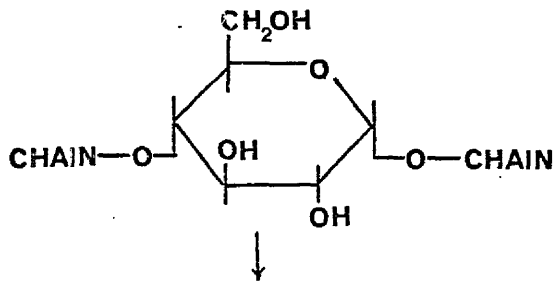
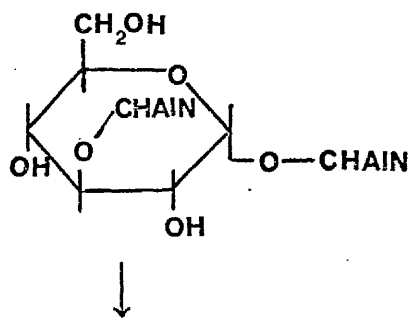
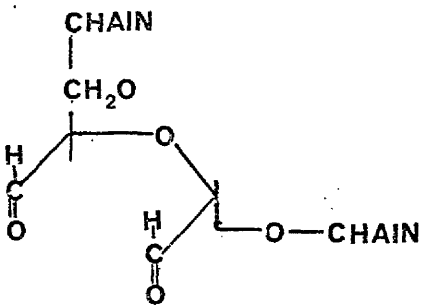
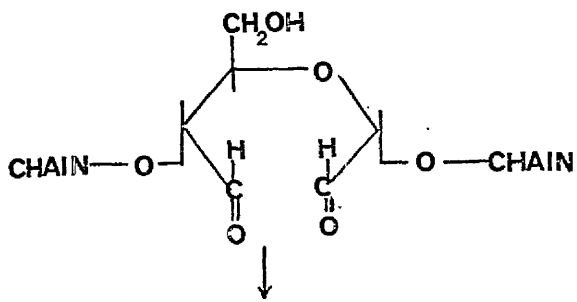
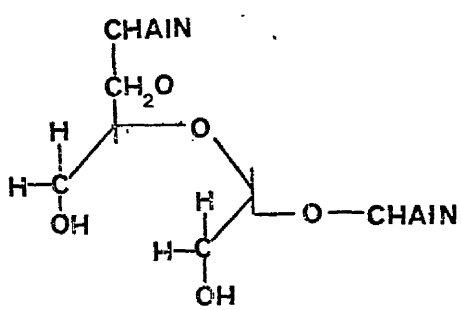
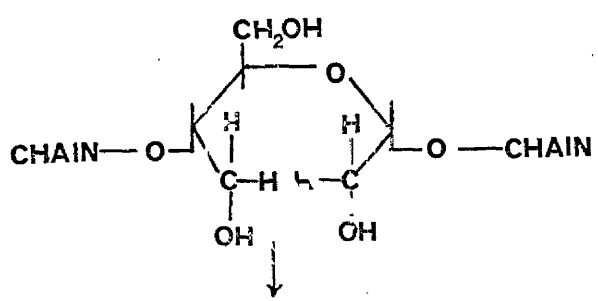
(i) Periodate oxidation. Periodate oxidises the bond between vicinyl hydroxyl groups. If one of the hydroxyl groups is a primary one, formaldehyde is released. If there are three adjacent hydroxyl groups, the middle carbon atom will be liberated as formic acid. The oxidation products of periodate oxidation of α-(1→6) -, α-(1→4)- and α-(1→3) - linked dextrans are shown in Table 1.2. From this it can be seen that the presence of an α-(1→3) - linkage precludes the possibility of oxidation of that particular glucose unit. α-(1→6)- and α-(1→4)- linked molecules are oxidised by periodate however, the former consuming two molecules of periodate per glucose unit whereas the latter consumes only one.

Until recently (Stoudt and Nollstadt, 1974) there was no evidence to suggest the presence of α-(1→2)- linkages in dextrans produced by oral bacteria. Any such linkages would form glyceraldehyde as the final product after hydrogenation and hydrolysis but no formic acid during periodate oxidation and could therefore, be differentiated from α-(1→6)- linkages. The repeating unit in a dextran is considered to be an anhydrous glucose unit (AGU) due to its loss of a water molecule in the formation of the glucosidic bond.

Formic acid can be produced by an AGU in an α-(1→6)- linkage or as a non-reducing terminal AGU. Since it is not possible to distinguish between them, they are known as '1→6 - like'. Periodate consumption is measured with time. A known amount of periodate is present at zero time and at regular intervals, a sample is removed from the oxidation mixture. The periodate which has not been consumed is removed by addition of a known amount of arsenic trioxide. The excess arsenic trioxide is then titrated against standardised iodine. From this, the periodate consumption can be

TABLE 1.2 PERIODATE OXIDATION PRODUCTS OF α-(1→6)-, α-(1→4) AND α-(1→3)-LINKED GLUCANS.

The top line of the table represents the structure of the anhydroglucose unit and the type of linkage which links it to the main glucan chain. The second line represents the product of periodate oxidation of the anhydroglucose unit. The third line represents the product of reduction of the periodate oxidised unit and the last line indicates the characteristic fragment released upon hydrolysis of the reduced product. The final fragment may then be identified by gas-liquid chromatography or paper chromatography.

	α-(1→6)	α-(1→4)	α-(1→3)
ANHYDROGLUCOSE UNIT			
PERIODATE OXIDISED UNIT			NO CHANGE
HYDROGENATED FRAGMENT			NO CHANGE
CHARACTERISTIC FRAGMENT	GLYCEROL	ERYTHRITOL	GLUCOSE

calculated. Formic acid released is measured by titration against barium hydroxide.

The reactions can be summarised as follows:-

$$NaIO_4 + 2KI + H_2O \longrightarrow NaIO_3 + 2KOH + I_2$$

$$2KOH + 2NaHCO_3 \longrightarrow Na_2CO_3 + K_2CO_3 + 2H_2O$$

$$I_2 + Na_3AsO_3 + H_2O \longrightarrow Na_3AsO_4 + 2HI$$

Thus:-

$$\% \quad 1\rightarrow 6 \text{ -like linkages} = \frac{\text{m mol HCOOH produced.}}{\text{m mol AGU}} \times 100$$

Where there are no α-(1→4) - linkages in the molecule 'mmolHCOOH produced' can be replaced by '$\frac{1}{2}$ periodate consumed'.

If α-(1→4) - or α - (1→2) - linkages are present:-

$$\% \ \alpha \ \underset{\text{linkages}}{1\rightarrow 2 \text{ or } 1\rightarrow 4} = \frac{(\text{m mol } IO_4^-) - 2 \times (\text{m mol HCOOH})}{\text{m mol AGU}} \times 100$$

The percentage of α- (1→3) - linkages can then be determined by difference such that all linkage types present add up to 100%. Over-oxidation problems can occur, but these can be minimised by careful choice of experimental conditions. Identification of the products of oxidation is desirable (Dyer, 1956) to rule out the possibility of over-oxidation. (1→6)- linked polysaccharides are fairly resistant to over-oxidation and (1→3) - linked polysaccharides are oxidised more slowly than (1→4) - linked.

(ii) Smith degradation. The degradation products obtained upon reduction and hydrolysis of the periodate oxidised dextrans are shown in Table 12. Identification of these can be used to confirm the dextran structure.

(iii) Methylation analysis. In this procedure the hydroxyl groups of the individual glucose units of the polymer are methylated. The glucan is then hydrolysed to release the methylated units which are identified, usually by gas-liquid chromatography, and the proportion of each type found. The

non-methylated carbon atoms of the derivatives indicate that the hydroxyl group attached to that atom was involved in bonding and was, therefore, shielded from methylation, e.g., 2, 3,4 tri-methylglucoside is derived from a glucose unit involved in (1→6) - linkages and there were no hydroxyl groups on the C1 and C6 portion available for methylation. Thus the proportions of linkage types and degree of branching may be found.

(iv) Enzymic degradation of dextrans. Dextranase [α (1→6) - glucan hydrolase], mutanase [α -(1→3) - glucan hydrolase] and α-amylase have all been used in attempts to hydrolyse the dextran in plaque (Guggenheim et al, 1972) and dextran samples (Guggenheim, 1970) in order to gain some information about the susceptibility of the dextran to enzymic attack and hence its structure. However, several flaws in this work are apparent. Initially, it was assumed that the dextrans in plaque would be predominantly α- (1→6) - linked and that all of the dextran would be hydrolysed (Minah et al, 1972).

The enzyme was known to bring about rapid hydrolysis of commercially prepared straight chain α- (1→6) -linked dextrans with the release of isomaltose. It was not realised that a highly branched dextran containing linkages other than α- (1→6) would not be completely hydrolysed (Hutson and Weigl, 1963). The specificity of the dextranase was unknown, it was often contaminated with other enzymes (Kelstrup et al, 1973) and, being of fungal origin, it may have little effect upon bacterial dextrans (Newbrun, 1972). In fact, a comparison of several commercially available dextranases by Tsuchiya et al (1952) showed marked differences in their ability to hydrolyse test dextrans (Ceska et al, 1972, Newbrun, 1972, Fitzgerald et al, 1968). Secondly, the commercially available dextrans are usually readily soluble in water and form homogeneous solutions such that the dextran is readily available to the enzyme. In plaque, however, the dextrans are

largely insoluble and their rate of hydrolysis must, therefore, depend upon the total surface area of dextran available to the enzyme and the area occupied by the enzyme (Kelstrup et al, 1973). The enzyme molecule, being very large, would not be able to penetrate plaque readily and would experience steric hindrance by a large, highly branched dextran molecule. Thus it may not be able to hydrolyse the less accessible linkages of the molecule (Hutson and Weigl, 1963). The method of extraction of the dextran molecule could also affect its structure in such a way as to alter its susceptibility to the enzyme. This has been shown to be the case with an α- (1→3) - linked glucan extracted from the cell walls of Schizophyllum commune by Wessels et al (1972).

Thirdly, the examination of dextran hydrolysates for isomaltose rarely revealed more than a trace (Gold et al, 1973, Minah et al, 1972) and this led to the conclusion that there was no dextran in plaque. However, the plaque was only exposed to dextranase for periods of 15-30 min. which, in the light of the above observations, could hardly be expected to allow very much hydrolysis to occur (Tanzer et al, 1972, Minah et al, 1972, Newbrun, 1972). The techniques used to identify isomaltose were also rather crude e.g. paper chromatography, and could not be expected to detect the very small amounts of isomaltose which would be released in such a short incubation period. Similar problems of penetration of the plaque, enzyme specificity and identification of the products existed with the use of mutanase.

(v) Infra-red spectroscopy. A thorough study of infra-red spectra produced from dextrans of Leuc. mesenteroides has been carried out by Heyn (1974). Only the region below 1400 cm^{-1} in the spectrum is specific for glucans. Variations in the sharpness of peaks or displacement of peaks can occur, depending on the origin and treatment of the dextran samples, e.g. peaks in the region 400 - 750 cm^{-1} can be displaced by varying the heating or drying of samples. Thus, the preparation of samples must be considered when comparing i.r. spectra.

Some peaks may be superimposed e.g. a peak at 800 cm^{-1} thought to be due to the presence of bound water is often difficult to distinguish from a peak at 790 cm^{-1} which is attributed to α-(1→3) branching linkage vibration. The spectra of dextrans of different molecular weights do not differ essentially.

Information can be obtained from i.r. spectra in respect of hydrogen bonding and bound water as well as the presence or absence of various linkage types and ring structures (Section 1.3.6).

I.r. cannot, however, supplant classical chemical methods.

Some of the information which has been obtained about the structure of various dextran preparations is given in Table 1.3. It demonstrates the changes which occur in dextran structure when prepared from progressively further purified dextransucrase preparations. Studies on dextran structure have also been carried out by Stoudt and Nollstadt (1974), Long (1971), Long and Edwards, (1972).

1.3.5 Physical properties of dextran.

Native dextran produced by Leuc.mesenteroides is a highly branched molecule of high molecular weight which is insoluble in water and stable to oxygen (Ebert and Schenk, 1968).

It is a glutinous, tenacious substance and it owes this property to the flexibility of intermolecular attractions. These consist of hydrogen bonds between hydroxyl groups but they are less perfect, more variable in strength and fewer in number than those found in strong, rigid polymers such as cellulose (Heyn, 1974).

Low molecular weight dextrans of Leuc.mesenteroides have 0·7 ~ 0·9 molecules of bound water per hydroxyl group whereas high molecular weight dextrans have 0·5. This is thought to be due to random coil formation by high molecular weight dextrans allowing intramolecular hydrogen bonding to occur and thereby, leaving fewer hydroxyl groups available to water. Removal of this water leads to a collapse of the molecule into an irregular conformation with lower flexibility than in solution (Heyn, 1974).

Hydration water in 'organised' form may occur in dextran such that water molecules associated with hydroxyl groups and with themselves form chains of water with strong hydrogen bonding.

Water molecules bound to two hydroxyl groups to form a bridge give the strongest hydrogen bonding of all and may also occur in dextran. Heyn (1974) proposed that every second oxygen atom of the chain is hydrogen-bonded to an hydroxyl group at an equal position in subsequent pyranose rings of a dextran molecule while intermediary oxygen atoms are bound to hydroxyls of a neighbouring dextran molecule, binding the two together over a certain length. Some hydroxyls are then left unbonded. Observations of Johnson et al (1974) endorse this view. They found that protofibrils of dextran were associated in pairs, when examined under the electron microscope (Section 1.3.6).

The tenacity of dextran is of obvious importance in plaque formation (Section 1.2.4).

It has also been suggested that negatively charged groups occur in dextran, setting up repulsive forces within the molecule (Rölla and Mathieson, 1970). However, the dextran studied was not absolutely pure and this effect could be due to contaminants. These contaminants might be ions which are reversibly bound to dextran (Rorem, 1955, Kelstrup and Funder-Nielson, 1972). Repulsive forces would prevent diffusion of charged molecules, such as acids, through the dextran while allowing passage of sugars. This could be of great importance in plaque, leading to the build-up of acids within plaque but allowing the passage of nutrients to bacteria.

Observations by Saxton and Critchley (1970) that sucrose and glucose diffuse readily through plaque but that some bacteria, particularly those furthest from the saliva interface, show signs of amino acid deficiences would tend to support this view.

Certain divalent ions, such as calcium, may form 'bridges' by combination with negatively charged groups, eliminating repulsion. Proteins may also be incorporated into dextran by combination with negative groups or by divalent cation bridges (Kelstrup and Funder-Nielson, 1972).

Gibbons and Banghart (1967) have shown a non-specific precipitation of dextran with protein and that a precipitate containing carbohydrate, protein, phosphorus and calcium forms in the presence of saliva. Kelstrup and Funder-Nielson (1972) observed a similar incorporation of lysozyme into insoluble dextran. They also found that dextran preparations, even after extensive purification, contained 0·018 - 8·1% (w/w) phosphate.

Rorem (1955) suggested that dextran takes up ions from the environment and an exchange of ions occurs between bacterial cells and polysaccharide. The polysaccharide then exchanges ions with those in the environment and thus a supply of ions can occur through the dextran to the plaque bacteria.

Thus the precipitation of protein/dextran complexes is probably of importance in plaque formation while the supply of essential nutrients to the bacterial cells is not seriously retarded by the presence of dextran.

1.3.6 The physical appearance of dextran. Examination of dextrans by electron microscopy has revealed differences between the polymers produced by different organisms (Johnson et al, 1974) and by the same organisms at different times after sucrose rinses (Johnson et al, 1974, Critchley and Saxton, 1970, Saxton and Findlay, 1971). Studies on S.mutans GS-5 which forms a deposit on the walls of the vessel in which it is grown in the presence of sucrose and S.mutans GS-511 and GS-514, mutants which do not form deposits, have shown differences in the polysaccharides elaborated from sucrose by the three organisms. GS-5 had a greater tendency to form bacterial aggregates and two types of polysaccharide were evident - a globular form and a fibrillar form which was found to consist of two proto-fibrils, 2nm wide, lying side by side (Section 1.3.5i). GS-511 formed a soluble globular polysaccharide which was only occasionally visible on the cell surface. GS-514 produced chains of globular polysaccharide which were replaced as the medium gelled by long fibrils consisting of two proto-fibrils 5nm wide.

Johnson et al (1974) concluded that the fibrillar polysaccharide was probably responsible for the ability to adhere to solid surfaces.

Newbrun et al (1971) observed that insoluble glucans prepared from different oral bacteria were spherical or ellipsoidal and frequently formed aggregates or beaded fibres and, occasionally, short chains of up to nine molecules. The molecules were 24.8 - 28.5 nm wide and 4.1 - 5.2 nm high which is of the same order as globules of polysaccharide observed by Johnson et al (1974). Cotton et al (1973) also observed globular and fibrillar forms of dextran which were produced by S.mutans in sucrose broth. The cells were completely submerged in polysaccharide within 72h and the enamel beneath the cells was found to be etched.

Bulkacz et al (1974) have shown that mutants of S.mutans E49 and 6715 which could not form plaque had a different dextransucrase content from the 'wild' type.

1.4 DEXTRANSUCRASE.

1.4.1 Mode of action.

$$(\text{Glucose})_n + y\text{Sucrose} \rightarrow (\text{Glucose})_{n+y} + y\,\text{Fructose}$$

Dextran Dextran

The reaction proceeds according to the above equation with donation of the glucosyl moiety of sucrose to an acceptor molecule and release of fructose (Eisenberg and Hestrin, 1963). The fructose, released as fructofuranoside, converts to the more stable fructopyranose form, pushing the equilibrium to the right so that the reaction is irreversible (Brock - Neely, 1960).

The enzyme is specific for sucrose which possesses sufficient energy in the glucose-fructose bond (- 6,600 cal/mol) for the formation of dextran (Newbrun, 1969).

A report was published of the formation of dextran from raffinose, maltose, lactose and glucose by Lactobacillus casei 32 - 1 + but the 'dextran' produced was not characterised and the carbohydrate assay was not specific for dextran. It seems likely that the substances produced were contaminating materials from the growth medium or other carbohydrates produced from the sugars (Hammond, 1969). Acceptor molecules such as sucrose, isomaltose, maltose and glycerol produce only short chain oligosaccharides and, this leads to a rapid increase in the rate of production but low yields of high molecular weight dextran (Ebert and Schenk, 1968, Koepsell et al, 1953, Newbrun and Carlsson, 1969).

Walker (1972) has shown that the glucose moiety is probably transferred to the C-6 position initially and that transfers to C-3 probably occur later.

This is borne out by experimental data (Table 1.3).

(i) Chain elongation. There are two possible mechanisms of chain elongation:-

a) Stepwise propagation. Here an enzyme-substrate complex donates the glucose moiety to the growing polymer with release of fructose.

b) Insertion. In this case an enzyme-substrate-polymer complex is formed which releases fructose. Hence the enzyme and polymer are bound together but the bond between them must be strong enough to hold during the reaction but flexible enough to allow insertion.

If the average molecular weight of the dextran is independant of the conversion rate, the latter mechanism almost certainly is in operation. Ebert and Schenk (1968) have shown this to be the case for dextran production by Leuc.mesenteroides. They also felt that branching occurred when an already elaborated dextran chain acted as acceptor but others (Brock-Neely, 1960) feel that a second, branching enzyme may be involved in some organisms. Brock-Neely (1960) observed that the molecular weight of dextran sometimes continues to increase after all the sucrose has been utilised. He postulated that preformed molecules were being attached to the main chain by a branching enzyme.

(ii) Enzyme requirements. Leuc.mesenteroides has been reported to require acetate for dextran production while Lactobacillus RWM-13 also requires Mn^{2+} and citrate (Dunnican and Seeley, 1963). No such requirements have been reported for dextran production by oral bacteria.

(iii) Induction. The dextransucrase of Leuc.mesenteroides is inducible with sucrose. Possibly the fructofuranose part of the sucrose molecule is responsible for the induction process (Brock-Neely and Nott, 1962). Dextransucrase producing plaque organisms studied have been found to produce the enzyme constitutively (Carlsson et al, 1969, Osborne et al, 1974, Chludzinski et al, 1974, Fukin et al, 1974).

Table 1.3 THE STRUCTURAL EXAMINATION OF STREPTOCOCCAL DEXTRAN PREPARATIONS

Organism	Dextran	Method of Examination	Structure
S. mutans Ingbritt	Water insoluble. Soluble in 0·5M NaOH	Periodate oxidation	34% α-(1→6) - like High degree of branching Baird et al, 1973.
S. sanguis 804	Crude enzyme preparation-dextran precipitated by 55% ethanol	Methylation analysis.	8·1% Gp- (1→ 82·4% →6)- Gp-(1→ 2·4% →3)- Gp-(1→ 7·1% →6 →3)-Gp-(1→
	As above but enzyme purified by hydroxylapatite chromatography.		12·9% Gp- (1→ 62·1% →6)- Gp-(1→ 10·8% →3)- Gp-(1→ 14·2% →6 →3)- Gp-(1→
	As above but enzyme further purified by isoelectric focussing.		14·9% Gp-(1→ 52·2% →6)- Gp-(1→ 17·7% →3)- Gp-(1→ 15·1% →6 →3)- Gp-(1→ Ceska et al, 1972.
	As above	Periodate Oxidation	49·8% (1→6) - like Guggenheim, 1970.
S. mutans OMZ 176	Water insoluble dextran.	Periodate Oxidation	15·2% (1→6) - like Guggenheim, 1970
	Enzyme purified by hydroxylapatite chromatography		16% (1→6) - like Newbrun, 1972.
	Purified enzyme of isoelectric point 5·6	Methylation analysis	40% α-(1→6)- 49·9% α-(1→3)- 5·0% α-(1→3)- branching.
	As above. Isoelectric point 5·0		2·9% α-(1→6)- 94·2% α-(1→3)- Ceska et al 1973.

(iv) Inhibition. Inhibition of dextransucrase has been reported by low molecular weight dextrans and glycerol (Newbrun et al, 1974, Gibbons and Nygaard, 1968) which act as acceptors giving a faster rate of reaction but a lower yield of high molecular weight dextran.

It has also been inhibited by N-bromosuccinimide, a reagent commonly used to oxidise the indole ring system in assays for tryptophan in proteins (Callaham and Heitz, 1974), which is believed to destroy a tryptophan residue at the active site of the molecule and thus prevent the formation of dextran. EDTA has been shown to inhibit dextransucrase from S.mutans BHT (Balliet and Chang, 1974) and maltose and melixitose have been shown to be competitive inhibitors of dextransucrase from S.sanguis 804 (Newbrun et al, 1974). Newbrun et al (1974) also showed fructose, glucose, raffinose and stachyose to be non-competitive inhibitors.

2,4- dinitrophenol and sodium azide did not inhibit growth of Lactobacillus casei 32 - 14 but they did inhibit dextran formation. Simultaneously, lactic acid production by the organism was increased by 20% and it was postulated that dextran was uncoupled from the formation mechanism and that the sucrose was then metabolised with release of acid (Hammond, 1969). Sodium dodecyl sulphate inhibits dextransucrase (Dart et al, 1974) as do other ionic detergents (Jablonski and Hayashi, 1970). This is probably due to the dissociation of the enzyme into several enzymically inactive subunits (Dart et al, 1974) or, at higher concentrations, to the denaturation of the enzyme by a detergent.

1.4.2. Optimum conditions of dextran production.

(i) pH. The pH optimum of dextransucrase produced by Leuc.mesenteroides and Lactobacillus species is usually fairly well defined and lies in the region of 5.0 - 5.5 (Ebert and Schenk, 1968, Gibbons and Nygaard, 1968). However, dextransucrases produced by streptococci have a much wider pH optima. They usually retain most of their activity over the pH range 5 - 8 (Gibbons and Nygaard, 1968, Balliet and Chang, 1974, Guggenheim and Newbrun, 1969) and often have a 'shoulder' at pH 5 - 6 and a peak in the region of

pH 7 - 8 (Gibbons and Nygaard, 1968, Guggenheim and Newbrun, 1969).

As explained in Section 1.3.3 the enzyme properties vary with the organism of origin and the growth conditions used. Cell-associated enzyme has also been shown to differ from that of the extracellular enzyme of the same organism (Gibbons and Nygaard, 1968).

(ii) Temperature. Here also, the enzyme produced by plaque streptococci has a wider tolerance than those produced by other organisms. Leuc. mesenteroides dextransucrase has an optimum in the region of 30°C (Dunnican and Seeley, 1963, Ebert and Schenk, 1968), whereas that of streptococci is more tolerant, remaining active in the region 30-50°C (Guggenheim and Newbrun, 1969, Chludzinski et al, 1974, Carlsson et al, 1969).

The presence of carbohydrate contaminating the enzyme preparation appears to confer greater temperature stability upon it (Long, 1971).

(iii) Sucrose concentration. Plaque streptococci tend to produce optimum amounts of dextran at higher sucrose concentrations than other bacteria and the amount of dextran produced continues to increase with the sucrose concentration over a longer period. The optimum usually lies within 5-10% sucrose (Cybulska and Pakula, 1963, Dunnican and Seeley, 1965) compared with 2% for Leuc. mesenteroides.

1.4.3 Enzyme production patterns. Very little is known about the production of dextransucrase in plaque and most work has been carried out on pure cultures of plaque bacteria. However, as has already been mentioned, conditions of production of the organism vary so widely, it is difficult to make comparisons (Section 1.3.3).

The enzyme is generally produced right through the growth of the culture but reaches a maximum during stationary phase (Carlsson et al, 1969, Long, 1971) when all glucose has been utilised.

It is possible that more than one dextransucrase may be produced as different preparations of enzyme from one organism produce dextrans with different properties, e.g., the extracellular enzyme of a plaque-forming bacterium

produced by Gibbons and Nygaard (1968) when incubated with sucrose at pH 8 produced a dextran which was >90% water-insoluble. The same enzyme preparation when incubated at pH 5 - 6 with sucrose produced dextran which was 59% water-insoluble. Cell associated enzyme from the same organism produced 90% insoluble dextran over the pH range 5 - 8. The latter preparation had a pH optimum at 7·0 while the former exhibited a shoulder at pH 5 - 6 and a peak at pH 8·0. Similar examples have been reported by other workers (Ceska et al, 1972).

It has also been observed that in dextransucrase production by Leuc. mesenteroides (Smith, 1970-71), S.bovis (Gold et al, 1973) and by S.mutans SL-1 (De Leon et al, 1974), a soluble dextran is produced first and this appears to act as a precursor to the formation of insoluble dextran.

1.4.4 Purification of Dextransucrase. In recent months, many investigators have attempted purification of dextransucrase from oral streptococci but most have achieved only limited success. With the exception of one group (Fukui et al, 1974), they have not succeeded in purifying the enzyme to homogeneity and Fukui et al (1974) only achieved this with one of two distinct enzymically active components produced by S.mutans HS-6. The techniques employed in the purification of the enzyme are listed below with a discussion of the problems encountered.

In these studies the organisms used produced dextransucrase as a constitutive, extracellular enzyme which simplified the procedure considerably as the cells could be centrifuged down and the culture supernatant used as the crude enzyme preparation (Chludzinski et al, 1974, Carlsson et al, 1969, Fukui et al, 1974, Osborne et al, 1973, Pitts and Keele, 1973, Guggenheim and Newbrun, 1969). As mentioned earlier (Section 1.4) some of these organisms are known to produce a small proportion of the enzyme intracellularly or in cell-bound form (Sharma et al, 1974, Gibbons, 1969) but few studies have been carried out on these enzymes. For inclusion of intracellular enzyme, it would be necessary to disrupt the cells, usually by sonication, to release

the enzyme (Gibbons, 1969, Gibbons and Nygaard, 1968) which would lead to the release of many other cell components into the supernatant to contaminate it and further complicate the purification procedure. Some of these enzymes may be released upon the dead and subsequent lysis of cells in batch culture.

The constitutive nature of the enzyme precludes the necessity to add sucrose to the bacterial culture. The sucrose would be converted to dextran which binds very strongly to the enzyme. It is extremely difficult to remove all the carbohydrate from the enzyme preparation and this has proved to be a problem with the purification of dextransucrase from other organisms, e.g., Leuconostoc (Brock-Neely and Nott, 1962).

The presence of other extracellular, constitutive enzymes which utilise sucrose as substrate has also proved to be a problem in the purification of streptococcal dextransucrase. These enzymes - levansucrase and invertase - interfere with dextransucrase assays and have proved to be difficult to separate (Fukui et al, 1974, Osborne et al, 1974). These enzymes are known to be produced by most dextran-producing streptococci with the exception of S. sanguis (Osborne et al, 1974, Carlsson, 1970).

In most cases, the first step in the purification of dextransucrase involves precipitation with $(NH_4)\ SO_4$ or acetone or adsorption onto hydroxylapatite.

(i) Precipitation with $(NH_4)\ SO_4$ or acetone. This is a lengthy and laboricus procedure followed by a lengthy dialysis step to remove all the salt from the enzymically active fraction (Osborne et al, 1973, Balliet and Chang, 1974). It probably leads to the loss of a large proportion of the enzyme due to the length of time it is stored at $4^{o}C$ and the amount of handling it receives (Carlsson et al, 1969). However, levansucrase activity may be removed at this stage (Chludzinski et al, 1974).

(ii) Adsorption onto hydroxylapatite. In this procedure a slurry of hydroxylapatite in buffer of low ionic strength and a pH of 6·8 is added directly to the culture supernatant (Carlsson et al, 1969, Guggenheim and Newbrun, 1969). Acidic and neutral proteins bind to the calcium of hydroxylapatite and basic proteins combine with the phosphate groups (Bernardi et al, 1972). The hydroxylapatite can then be centrifuged down and the supernatant discarded. However, the ionic strength of the medium may be sufficiently high to prevent adsorption of all the enzyme, in which case dialysis against the equilibrating buffer may be a necessary first step. The enzyme is then eluted from the hydroxylapatite with a molarity gradient using sodium or potassium phosphate buffer. The phosphate competes with the acidic proteins for the calcium and the sodium or potassium competes with the basic proteins for phosphate (Bernardi et al, 1972). Sodium phosphate buffers of the molarity required for elution of dextransucrase (approx. 0·5M) tend to crystallise out at 4°C and the potassium salt must be used in its place.

The enzyme is eluted over a fairly wide phosphate concentration range but a large amount of contaminating protein is removed (Carlsson et al, 1969). A batch process has been used which is fairly laborious but column chromatography would give finer control of the buffer concentration and involves smaller volumes of liquid. However, hydroxylapatite can present difficulties in maintaining the flow rate as it is an extremely fine powder which packs down firmly.

Again, enzyme losses may occur through repeated handling and lengthy storage at 4°C (Carlsson et al, 1969).

(iii) Isoelectric focussing. This procedure has been widely used in the purification of dextransucrase (Chludzinski et al, 1974, Carlsson et al, 1969, Newbrun, 1971, Guggenheim and Newbrun, 1969) but has several attendant problems. Density gradients using sucrose cannot be used as the enzyme

converts it to dextran and, as glycerol inhibits dextransucrase (Section 1.4.1.iv), its use makes it necessary to carry out a very careful dialysis step before the enzyme activity can be assayed (Chludzinski et al, 1974). The enzyme was, in some cases, seen to precipitate out during isoelectric focussing but this could be prevented to some extent by the addition of 2M urea or 1·66M glycine to the enzyme preparation (Carlsson et al, 1974). A considerable amount of the enzyme activity applied to the column was lost (Carlsson et al, 1969, Chludzinski et al, 1974). This may have been due to the separation of the enzyme into many enzymically active fractions, some of which were too small to be recovered. Chludzinski et al (1974) obtained at least ten bands of enzyme activity on polyacrylamide gel electrophoresis of the protein eluted from hydroxylapatite chromatography but recovered only four bands after the protein from hydroxylapatite chromatography had undergone isoelectric focussing. Carlsson et al (1969) also observed precipitation of enzymically active protein from a S.sanguis dextransucrase preparation upon dialysis.

In most cases, three or four distinct bands of enzyme activity are discernable from dextransucrase preparations after isoelectric focussing (Carlsson et al, 1969, Chludzinski et al, 1974, Osborne et al, 1974) but as many as seven have been reported from preparations of S.mutans dextransucrase (Guggenheim and Newbrun, 1969).

(iv) Chromatography on dextran gels. This technique has been used in attempts to separate dextransucrase on the basis of its molecular weight (Long, 1971, Pitts and Keele, 1973). However, dextransucrase binds very strongly to dextran and some enzyme may be lost in this way. The enzyme can also be contaminated by dextran which is extremely difficult to remove (Long, 1971).

(v) Chromatography on DEAE-cellulose. This technique has also proved to be useful in dextransucrase purification (Pitts and Keele, 1973, Fukui et al, 1974) particularly from S.mutans cultures as Fukui et al (1974)

were able to use it to separate dextransucrase from invertase.

(vi) Sodium dodecyl sulphate gel electrophoresis. This method was used by Fukui et al (1974) to determine the molecular weight of the dextransucrase fraction they purified but Dart et al (1974) found that two dextransucrase preparations of different isoelectric points dissociated into six subunits each which did not possess enzymic activity. The molecular weight of the enzyme purified by Fukui et al (1974) was 170,000. Thus the small enzyme may only consist of one sub-unit while the larger enzymes consisting of six sub-units may lose their activity on dissociation because the active site is destroyed by this process.

1.4.5 The Active site of Dextransucrase. The only reported studies on the active site of dextransucrase have been conducted on partially purified enzyme from S.sanguis 804 (Carlsson et al, 1969) by Callaham and Heitz (1973). Rose bengal, a halogenated fluorescein dye implicated in the photo-oxidation of tryptophan and histidine was incubated with dextransucrase under illumination. Photo-oxidation of the enzyme occurred.but the presence of dextran protected the dextransucrase. Eosin yellowish combined with the enzyme in competition with sucrose and it was suggested that the site of photo-oxidation was in close proximity to the substrate and acceptor binding sites. More recent work has suggested the presence of a tryptophan residue in the active site of the enzyme (Callaham and Heitz, 1974) as the conditions under which photo-oxidation occurs are very similar to those of tryptophan oxidation. N-bromosuccinimide, which destroys the indole ring of tryptophan, and hydroxy-5-nitrobenzyl bromide which is also selective for tryptophan both inhibit dextransucrase.

1.5 Plaque ecology.

The formation and development of dental plaque has been described (Section 1.2). An understanding of the role of each plaque constituent in the overall ecology of plaque is necessary to understand the processes which lead to the initiation of dental caries.

TABLE 1.4 PURIFICATION OF DEXTRANSUCRASES OF PURE CULTURES OF PLAQUE BACTERIA

Organism	Cultural Conditions	Purification Procedure	Yield	Properties
Strep. mutans. 6715	Overnight growth with sucrose. No pH control.	$(NH_4)_2 SO_4$ precipitation Hydroxlapatite chromatography Isoelectric focussing. (Chludzinski et al, 1974)	13% (1500-fold purification)	Two distinct protein bands obtained. One (containing 90% of enzyme activity) studied further – mol. wt. = 94,000. Temperature optimum : 34–42°C. Ip 4·9 pH optimum : 5·5(4·2–6·0) Km 3m mol. sucrose.
Strep. mutans. FA1	24–36h growth with glucose. No pH control.	$(NH_4)_2 SO_4$ precipitation. Chromatography on Sephadex G-75 (Long, 1971).	10%	Contaminated with 23% carbohydrate Mol. wt. >70,000. Very stable to temperature. pH optimum : 6·0(5 – 7).
Strep. mutans. CMZ 178	Anaerobic. Harvested at end of log phase with glucose pH control at 6.0	$(NH_4)_2 SO_4$ and acetone precipitation. Hydroxylapatite chromatography. Isoelectric focussing. (Guggenheim and Newbrun, 1969)		pI values of 4·24, 5·00, 5·65 plus four more components in small amounts. pH optimum 5 – 7. Temperature optimum 30–50°C Km values 0·98 – 7·23 m mol. sucrose.
Strep. mutans. JC2	Anaerobic	DEAE cellulose chromatography. Chromatography on Sepharose 6B. (Pitts & Keele, 1973)		Product – not homogeneous.
Strep. sanguis 34	Overnight growth with glucose. Intermittant pH control at pH 5.	Hydroxylapatite chromatography. Isoelectric focussing. (Carlsson et al, 1969).	15% (440-fold purification)	pI values of 7·9, 6·4 and 4·5. pH optimum 5·2 – 7·0. Temperature optimum 45°C. Km 4·8 m mol sucrose.
Strep. mutans NS-6	6h growth with glucose. Intermittant pH control.	$(NH_4)_2SO_4$ precipitation. Sepharose 6B. chromatography DEAE cellulose chromatography. Hydroxylapatite chromatography. (Fukui et al, 1974).		Two components on DEAE cellulose. One studied further. Single band on polyacrylamide gel and immunodiffusion Mol. wt. 170,000 pH optimum 5·75. Km: 2 m M sucrose. Produced soluble polysaccharide of 94% (1→6)- like linkages.

As it is the acid produced by plaque bacteria which causes the initial breach of the enamel surface, it is the production of the acid by bacteria and the retention of it within plaque which is of prime importance.

1.5.1 Acid production. Many of the bacteria isolated from plaque have been examined for their ability to produce acid and they have been found to produce copious amounts from a wide variety of sugars (Muhlemann and De Beaver, 1970, Stephan, 1938, Carlsson, 1965). In the presence of dietary sugars, therefore, one would expect the pH of plaque to fall very quickly. However, if the bacteria were not highly aciduric, the low pH would rapidly kill them and their ability to induce caries would cease. Plaque bacteria tend to be highly aciduric and some can continue to produce acid at pH values as low as 4 (Krasse, 1970, Stephan and Hemmens, 1947). These bacteria, predominantly lactobacilli (Strålfors, 1948) are therefore very important in the carious process. Most of these studies on the acid-producing ability of plaque organisms have been conducted on pure cultures of the test organism grown in sugar broths.

An additive effect has been observed when lactobacilli are grown with bacteria which do not normally produce a low pH (Stephan and Hemmens, 1947). The bacteria in dental plaque are present in very high numbers (Tanzer et al, 1969) growth is slight (Tanzer et al, 1969), the nutrients are continuously being replaced and one would expect the acid produced to be diluted by oral fluids and washed away. It is, therefore, questionable as to whether it is valid to compare the acid production of pure cultures of rapidly growing bacteria in batch culture, where the bacterial numbers are relatively low, nutrient is limited and the acid is not removed, with acid production in plaque. Studies of acid production in undisturbed plaque have been extremely difficult. Introduction of an electrode into plaque necessarily disturbs it and causes changes within the 'micro-environment' and, until recently, it was difficult to produce a pH electrode small enough to measure the pH

within a thin layer of plaque. Incorporation of an electrode into a prosthetic device to be worn in the mouth which continually monitors the pH and transmits the measurements by radiotelemetry also has problems. Plaque which forms over a prosthetic device or over the pH electrode cannot be exactly the same as that which forms on an enamel surface (Section 1.2.2iii). Attempts to separate the electrode from plaque by an ultra thin layer of enamel would solve the problem but have met with great technical difficulties. Studies on pH changes of plaque in the presence of various sugars and foods have shown that the pH falls very rapidly (within 3 minutes) to values as low as pH 4 and remains low for up to 40 minutes before gradually returning to its initial level (De Boever and Muhlemann, 1969). Thus, it seems, that the acid builds up for a short while and then is gradually utilised or disperses. This build-up of acid is believed to be due to a diffusion barrier created by the matrix material of plaque, predominantly the dextran. Gradually the acid penetrates the barrier but diffusion is considerably slowed.

It is thought that the highly branched structure of dextran may trap the small acid molecules within it, perhaps in a similar way to that used in separating mixtures of components of different molecular weights in gel filtration columns. The presence of charged groups within dextran may also retard the dispersion of acid (Section 1.3.5).

Thus pools of acid build up over short periods in close proximity to the tooth surface, allowing the initial demineralisation of enamel to occur.

In the absence of dietary sugars, the bacteria metabolise storage polysaccharides which are produced during periods of high sugar concentration (Critchley et al, 1967). These polysaccharides are of two types - intracellular, glycogen-like polysaccharide and the extracellular levan. These, too, are metabolised with the release of acid so that acid production is continued even in the absence of food. However, in tube-fed animals in which the plaque bacteria rely entirely on stored polysaccharide, caries

incidence is low (Bowen and Cornick, 1970). However, it can be argued that, in the absence of food, the storage polysaccharide cannot be replenished and the role of storage polysaccharides in normal plaque cannot be ascertained. A positive correlation has been demonstrated between the number of intracellular polysaccharide producing bacteria and caries incidence (Gibbons, 1968, Saxton, 1969). Levan has a very rapid turnover in plaque (Higuchi et al, 1970). It constitutes approximately 1% of the dry weight of 'resting' plaque but can increase by as much as 300% after a sucrose rinse (McDougall, 1964). Da Costa and Gibbons (1968) found that 37% of plaque bacteria were capable of hydrolysing levan. Levansucrase is very difficult to separate from the cell which produces it and attempts to solubilise it have had little success (Sharma et al, 1973, Gibbons and Nygaard, 1968). However, an ecological advantage is given to cells able to surround themselves with a store of levan and release of the enzyme into the environment would give this advantage to other bacteria, increasing the competition.

1.5.2 Limitation of growth. The high concentration of bacteria in plaque must compete for space and food supplies and evidence suggests that it is limitation of space and not food supplies which limits the growth (Carlsson and Johansson, 1973). Cells which are coated in dextran do not grow as rapidly as uncoated cells (Tanzer et al, 1969) which may be due to the limitation of diffusion of substances to and from the cell by the dextran. Antagonistic effects have been demonstrated between plaque bacteria (Bartels, 1933, Hine 1935-36, Clough, 1934, Belding, 1948, Krasse, 1970). For example, S.sanguis has recently been shown to produce hydrogen peroxide, in the presence of even minute traces of oxygen (Rosan and Eisenberg, 1973). This substance limits the growth of many bacteria, including S.mutans (Holmberg and Hallander, 1973). As little as 0·2% (v/v) hydrogen peroxide has been shown to inhibit growth of S.mutans Ingbritt (Donoghue, 1974)

but bacteria which produce catalase e.g. Neisseria (Taylor and Juni, 1961) can break down the hydrogen peroxide and render it harmless. This mechanism may be of great importance to S. sanguis in the initial colonisation of the tooth, allowing it to slow down the colonisation of the tooth by other bacteria. Approximately 55% of the aerobic streptococci of 'young plaque' (3 - 7 days old) are 'sanguis-like' (Carlsson, 1966) but as plaque builds up conditions become anaerobic and the proportion of streptococci declines (Egelberg, 1971).

Thus it can be seen that plaque is a very complex mixture of components which are inter-related in an intricate, finely-balanced community. The conditions prevalent in a particular area of plaque may be quite different from another area but the variables within the micro-environment and between micro-environments are inter-related and alterations within any section could cause profound changes within the plaque.

1.6 Conclusions.

Dental plaque is an extremely complex mixture of components which build up upon the tooth surface, initiating with the precipitation and adsorption of proteins and bacteria onto the charged enamel surface. The bacteria proliferate and metabolise materials in the plaque and oral fluids, surrounding themselves with a carbohydrate matrix of dextran, levan and heteropolysaccharides. The deposition of substances and production of matrix material continues and metabolic acids gradually accumulate, trapped within the dense plaque material.

Dextran appears to play an important role in the formation and development of dental plaque and in maintaining its integrity. It may also assist in the transfer of ions from the environment to the bacteria, allowing diffusion of small molecular weight uncharged molecules such as sugars and delaying the dispersion of charged molecules such as bacterial acids. Large molecules such as bacterial enzymes may be trapped in an active form within the dextran. The build-up of acid within the plaque leads to the initiation of caries. Thus dextran plays an important role in the processes leading up to the initiation of caries and its elimination from plaque may be an important step towards the prevention of dental caries. Elimination of dextran, once formed, would be extremely difficult by chemical methods due to the problems of penetration of the dense bacterial accumulation and the highly branched nature of the dextran molecule. Mechanical methods, such as tooth-brushing, are probably the best methods known at present but this only removes part of the deposit which rapidly builds up again. It is not feasible to brush the teeth as often as would be necessary to prevent caries.

It seems more plausible to prevent dextran formation de novo. This could be done by eliminating all sucrose from the diet but such measures would neither be acceptable to the population nor would it be feasible to eliminate it entirely from food. However, prevention of dextran synthesis de novo

by inhibition of dextransucrase may provide the answer to this problem. However, dextransucrase production is far more complex than at first appreciated and each dextran-producing organism may produce several different dextransucrases, the pattern differing with the conditions prevalent at the time of production. Thus plaque may contain a wide variety of dextransucrases, each with slightly different properties from the next. It would, therefore, be necessary either to discover an inhibitory substance which is effective against all the dextransucrases or one which inhibits the enzyme/s which contribute most to plaque formation.

1.7 Aims of this study.

It was the aim of this study to investigate the production of dextransucrase by oral bacteria in order that the enzyme might eventually be purified and its physical and biochemical properties studied. It might then be possible to inhibit dextran production in the hope that such inhibition, in vivo, may prevent or limit plaque production and thus slow or stop the carious process.

A considerable amount of work has been carried out in other laboratories on dextransucrase production since the start of this study (see Section 1.4.4) and the full complexity of the problem has gradually emerged.

As discussed earlier in this section, different organisms produce different dextransucrases and any one organism may produce three, four or more distinct protein components with dextransucrase activity. The proportions of the enzymes may also vary according to the cultural conditions of the organism. There is also evidence to suggest that the dextrans produced by the different protein fractions may differ structurally.

Most of the work on dextransucrase has been conducted on that produced by S. mutans partly because this organism produces an adherent deposit in the presence of sucrose whereas S. sanguis does not. However, it is now recognised that the inability of an organism to produce a plaque-like deposit in pure culture does not necessarily reflect an inability to form plaque in

the presence of the mixed oral flora.

S.mutans was also chosen because it is considered to be highly cariogenic whereas S.sanguis is not generally considered to be cariogenic. However, S.sanguis makes up approximately 55% of aerobic streptococci in early plaque (Carlsson, 1965) and it is believed to be an important organism in the initiation of dental plaque formation. Recent work suggests that S.sanguis may be cariogenic (Duany et al, 1973) but again, the ability of an organism to induce caries may be affected by the presence of other bacterial species. S.sanguis does not produce invertase in the absence of sucrose or levansucrase which would complicate the purification procedure and work by Carlsson et al, (1969) suggests that purification of the enzyme is relatively simple, producing only three separate protein components.

It was, therefore, decided to examine dextransucrase production by S.sanguis with a view to determining the optimal conditions for its production prior to attempting its purification. Very little work has been conducted along these lines and it was decided to examine the production of the enzyme at different pH values as this would probably have a profound effect upon the activity of the enzyme.

It emerged from these studies, that the enzyme was produced in three distinct phases and it was decided to examine the dextran produced by the enzyme at each phase, in order to determine any structural differences.

It was not the intention of this study to determine the exact structure of the dextran but to highlight any differences which could be shown between the dextrans which might indicate differences in structure and hence in the enzyme at each phase.

The complex nature of the problem gradually emerged and the work therefore, took longer than expected. It was not possible in the time available, to achieve very much along the lines of purification of the enzyme. The purification and yield of the enzyme were rather low but experience gained in the preliminary experiments would probably ensure better results with further attempts.

2. METHODS

2.1 MATERIALS.

Laminarin and Nigeran were obtained from Koch-Light Laboratories, Colnbrook, Buckinghamshire; Amylopectin, Glycogen and α-Amylase were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A., and Dextran (T2000) from Pharmacia Fine Chemicals A.B, Uppsala, Sweden. Two preparations of Mutan were used: one was a gift from Schweizersche Ferment A.G., Basle, Switzerland, and the second was prepared from a sucrose containing supernatant of Streptococcus mutans OMZ 176, CBS 350.71 as described by Guggenheim (1969).

S-glucan was prepared from the mycelium of Aspergillus nidulans strain Eidam biA_1, kindly supplied by Dr. B. Cohen of the Department of Genetics, University of Glasgow.

Mutanase was a gift of Schweizersche Ferment A.G., and dextranase was purchased from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.

All other reagents were of 'AR' Grade, supplied by BDH Chemicals Limited, Poole, England.

2.2 MICRO-ORGANISMS

2.2.1 Streptococcus sanguis 804, NCTC 10904, an isolate from human dental plaque (Carlsson, 1968), was obtained as a freeze-dried culture from Professor J. Carlsson of the Department of Oral Microbiology, University of Umeå, Uppsala, Sweden. It was maintained on horse blood agar slopes (Section 2.3) at 4°C with monthly transfer.

2.2.2 Streptococcus mutans OMZ 175, CBS 350.71 described by Guggenheim (1969) was obtained as a freeze-dried culture from Dr. F.J. McKean of the Department of Oral Medicine, Glasgow Dental Hospital and School,

Glasgow. It was maintained on horse blood agar slopes at 4°C with monthly transfer.

2.3 MAINTENANCE MEDIUM

Blood agar base (40g) (Oxoid, Southwark Bridge Road, London) was added to distilled water (1 litre) and autoclaved at 103·5 kPa for 20 minutes. It was cooled to 40°C and defibrinated horse blood (Oxoid) was added aseptically to give a final concentration of 5% (v/v).

2.4 CULTURE MEDIA

2.4.1 Carlsson's diffusate medium (Carlsson et al,1969). This is a modification of the medium developed by Cybulska and Pakula (1963). Casein hydrolysate (25g) (Oxoid), tryptose (100g) (Oxoid) and yeast extract (50g) (Oxoid) were added to distilled water (500 ml) and dialysed (Section 2.13.1) overnight against 1·5 l distilled water. The dialysis was repeated twice more against the same volume of distilled water for 16h and the diffusates pooled. This liquid will be referred to as Carlsson's diffusate. The volume was made up to 4 l with distilled water and anhydrous K_2HPO_4 (15g) added. This solution was autoclaved at 103·5 kPa for 20 minutes. This will be referred to as Carlsson's diffusate medium.

D-glucose (50g) was added to distilled water (1 litre) and autoclaved at 103·5 kPa for 20 minutes. It was then added aseptically to the sterile diffusate to give a final glucose concentration of 1% (w/v).

2.4.2 Nutrient broth. Nutrient broth No.2 (Oxoid) (25g), was added to distilled water (800 ml) and autoclaved as above. 5% (w/v) sterile glucose solution (200 ml) prepared as above was then added aseptically.

2.5 GROWTH OF S.sanguis

Sterile Carlsson's diffusate medium (200 ml) (Section 2.4.1) in a 500 ml Ehrlenmeyer flask was incubated at 37°C with stirring.

2.5.1 Rate of growth and pH change of S.sanguis. Culture vessels set up as above were inoculated with 5 ml of a 16h starter culture of S.sanguis. 2 ml samples were removed aseptically at known time intervals and the pH measured. The change in turbidity (Section 2.15.1) with time was taken as a measure of the bacterial growth rate.

2.5.2 Dextransucrase production by S.sanguis. Cultures set up as above were sampled at intervals and assayed for cell growth and dextransucrase (Section 2.15.2ii).

2.6 ELUCIDATION OF OPTIMUM CONDITIONS FOR DEXTRANSUCRASE PRODUCTION

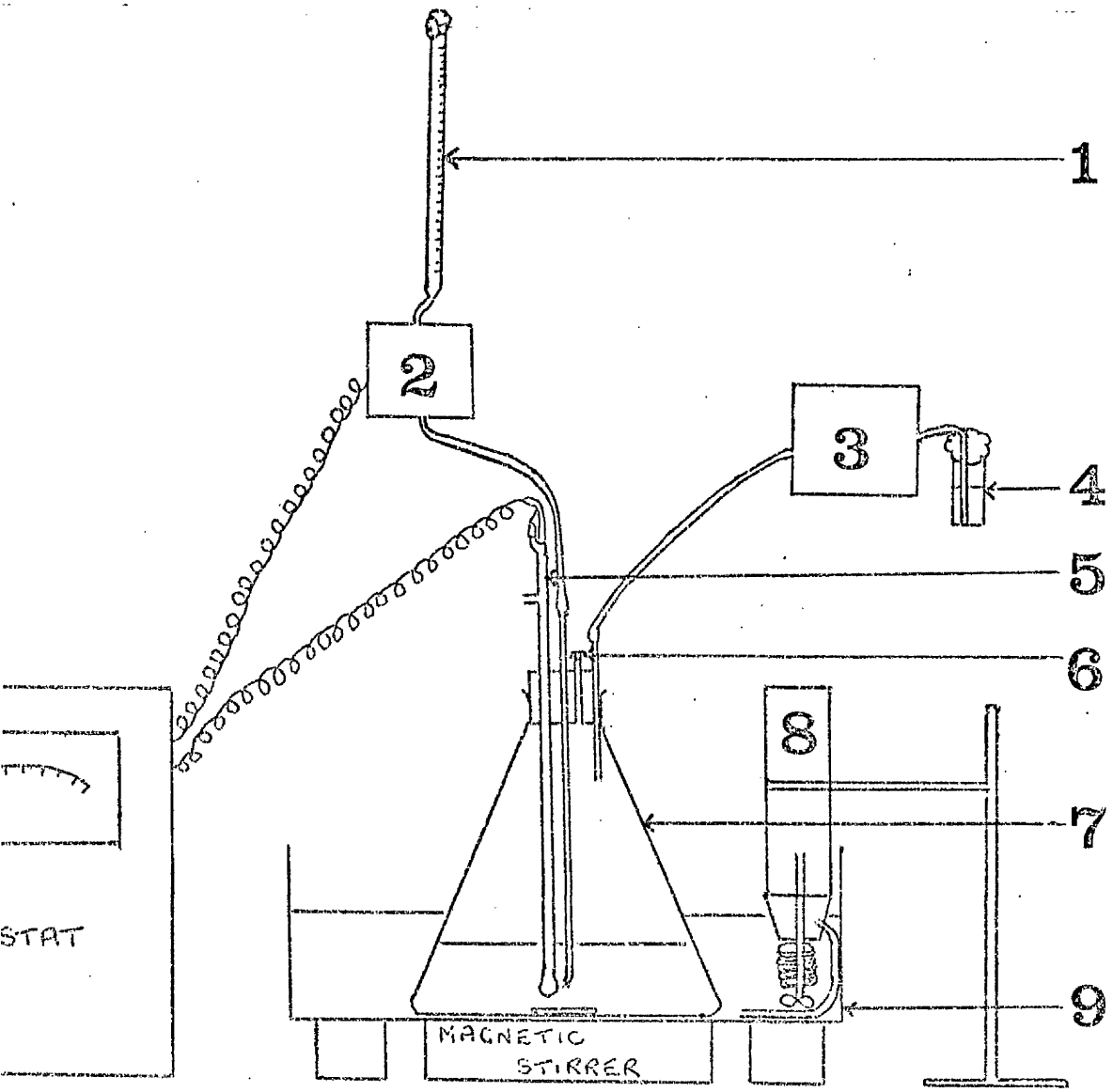
2.6.1 The effect of inoculum size. The effect of a 0·5% (v/v) and a 2·5% (v/v) inoculum of a 16h culture were examined by inoculating culture medium with 1 ml and 5 ml respectively of a 16h starter culture. Samples were assayed for cell growth and dextransucrase production (Section 2.15).

2.6.2 The effect of inoculum age. Cultures were set up as before using 5 ml of 7h, 10½h and 16h starter cultures respectively.

2.6.3 The effect of pH control. S.sanguis produces copious amounts of acid when grown in Carlsson's diffusate medium (Section 3.1.1) and it was decided to examine S.sanguis under conditions of controlled pH in order to determine any limiting effect of the acid on growth and dextransucrase production.

A 500 ml wide-necked Ehrlenmeyer flask was fitted with a rubber bung containing four ports (Figure 2.1). One port carried an autoclavable combination pH electrode (Activion Glass Limited, Mitchell Hall, Kinglassie, Fife) - No.5 on Figure 2.1, the second an inlet tube for NaOH, the third was attached to a Universal bottle (4) which served as an inoculation port and the last was a sampling port (6).

The flask contained Carlsson's diffusate (156 ml) and a magnetic stirring bar. The Universal bottle contained 4 ml of the same liquid. The apparatus was autoclaved at 103·5 kPa for 20 minutes.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STAT
MAGNETIC
STIRRER

Fig.2.1. Fermenter flask assembly for growth of S.sanguis under conditions of controlled pH.

A fermenter flask assembly was set up as shown above. The flask - 7 contained 156ml Carlsson's diffusate and a magnetic stirring bar and was sealed with a rubber bung. The bung carried an autoclavable pH electrode - 5 , an inlet tube attached by rubber tubing to a burette - 1 , a sampling tube - 6 sealed with a rubber septum and an inoculation port attached by rubber tubing to a Universal bottle - 4 containing 4ml Carlsson's diffusate. This entire assembly was autoclaved at 103·5 kPa for 20 minutes and set up in the water bath at 37°C as shown. The pH electrode was attached to the pH-stat and the desired pH selected. A peristaltic pump - 2 was attached to the pH stat and the NaOH inlet. When the pH fell below the desired level the pump was activated and metered in sterile M NaOH from the burette - 1 until the required pH was restored. Sterile glucose solution (5% w/v) 39 ml was added to the flask by means of a sterile syringe and needle by way of the sampling port and 1 ml was added to the Universal bottle. The Universal bottle was then inoculated with a loopful of S. sanguis and suspended in the water bath at 37°C. After 16 hours a peristaltic pump - 3 was activated by a timer and the contents of the Universal bottle were transferred into the flask. Samples were removed by means of a sterile syringe and needle by way of the sampling port. The water bath was maintained at 37°C by the water heater - 8 .

Sterile glucose solution (5% w/v) (39 ml) was added aseptically to the flask and 1 ml. to the Universal bottle.

The apparatus was then set up in a water bath at 37°C as in Figure 2.1 The pH electrode (5) was connected to the Radiometer Titrator type TTT1 (Radiometer A/S, DK 2400, Copenhagen N.V. Denmark, and the inlet tube for NaOH to a sterile reservoir (1) containing sterile NaOH. The flow of liquid from this reservoir is controlled by a peristaltic pump (2) which is connected to the titrator.

The Universal bottle (4) was inoculated with S.sanguis and suspended in the water bath at 37°C. The desired growth pH was selected on the titrator and the pH of the medium in the flask adjusted accordingly. After 16h growth a timer activated a second peristaltic pump (3) which transferred the contents of the Universal bottle into the Flask. When the pH subsequently fell below the desired level the titrator activated a pump (2), metering in sterile M NaOH until the desired pH was restored. Sterile syringes and 10in stainless steel needles (Hamilton Micromesure BV, The Hague, Holland) were used to remove samples at zero time and at hourly intervals. The flask was maintained at 37°C with constant stirring for up to 72h.

Experiments were performed at pH values of 5·0, 6·0, 7·0 and 8·0 and subsequently at 6·5 and 7·5.

2.7 TREATMENT OF CULTURE SAMPLES

Immediately upon removal of a sample, it was assayed for cell growth (Section 2.15.1). Every fourth sample was examined for contamination by foreign bacteria by inspection of a Gram stained slide and by plating out on blood agar plates.

It was then centrifuged at 1600g for 20 minutes at 4°C and the supernatant decanted. The cells were discarded and the supernatant assayed for glucose, protein and dextransucrase (Section 2.15.2ii, 5 and 6i).

2.8 BATCH CULTURE OF S.sanguis FOR DEXTRANSUCRASE PRODUCTION

A 12 l magnetic drive fermenter (Virtis Co. Inc., Gardiner, New York 12525) was set up as shown in Figure 2.2 with ports for an inlet for NaOH and an autoclavable pH electrode (Figure 2.2 No.5). A port with a self-sealing rubber septum (6) served as sampling port. A bottle (4) sealed with a rubber bung carrying an outlet tube and an air inlet tube fitted with a bacteriological air filter - 3 (Whatman Filter Tubes, Reeve Angel Scientific Limited, Gaunt Street, London) was used for inoculation and introduction of sterile glucose solution.

Carlsson's diffusate (6·24 l) was introduced into the fermenter which was then autoclaved at 103·5 kPa for 75 minutes. Sterile glucose solution 5% (w/v) (1·56 l) was added and the apparatus set up in a water bath at 37°C as shown in Figure 2.2.

The apparatus was connected to the titrator in the same way as the flask assembly (Section 2.6.3).

200 ml of a 16h culture of S.sanguis was added aseptically and sampling carried out at hourly intervals.

The pH was maintained at a constant value by addition of 10M NaOH. The removal of samples larger than 50 ml necessitated the addition of a siphon tube to the fermenter prior to autoclaving. Large samples could then be siphoned off aseptically.

2.9 PREPARATION OF GLUCANS

2.9.1 Mutan 2 was prepared from the supernatant of a culture of S.mutans OMZ 176 which had been grown in nutrient broth at 37°C for 24h (Guggenheim 1969). The cells were centrifuged down and the supernatant supplemented with penicillin (440 u/mg)/streptomycin (440 u/mg) mixture (BDH tissue culture media) to give a final concentration of 100 µg/ml in order to prevent bacterial

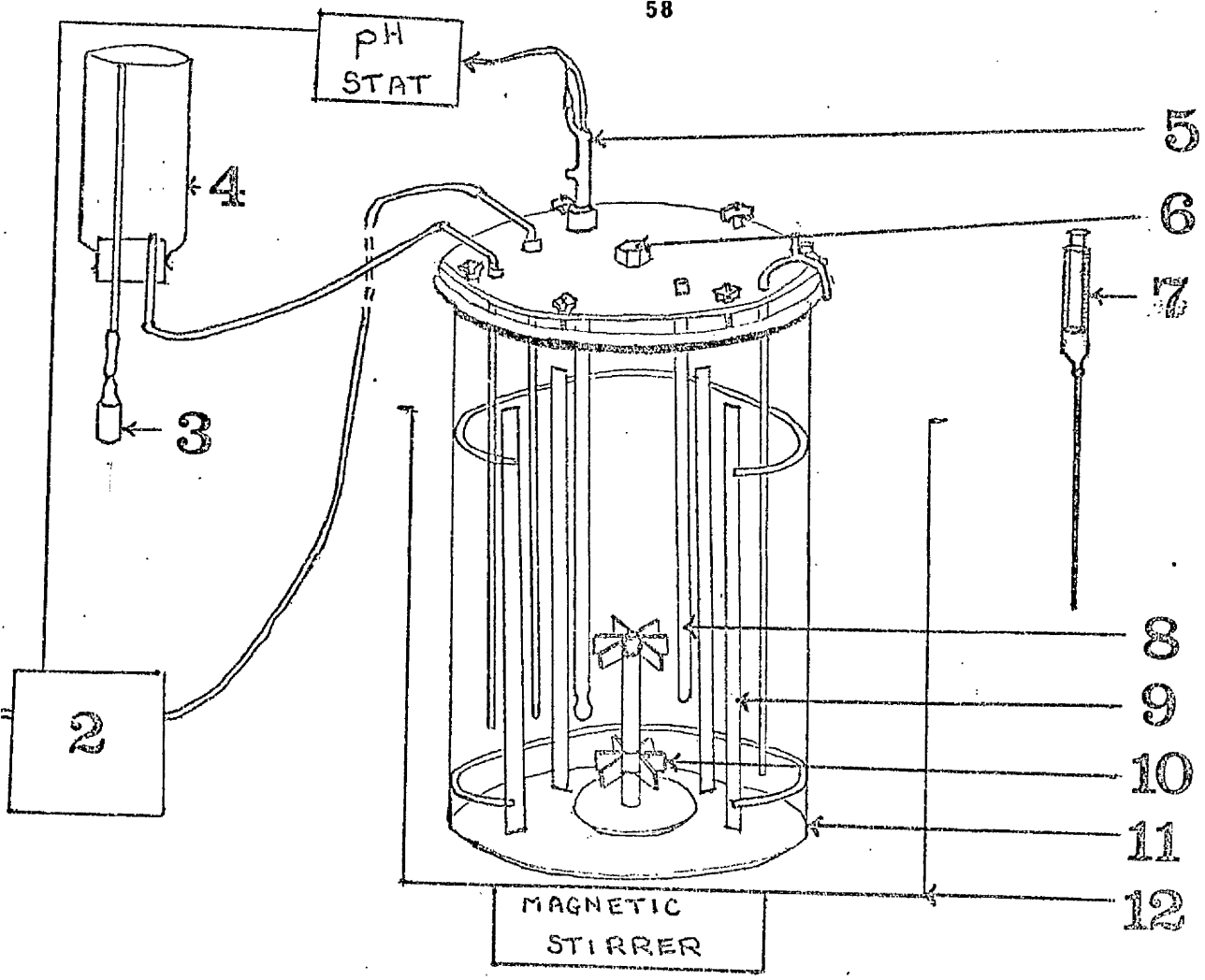


Fig.2.2 Fermentation Cell for Production of Dextransucrase from S.sanguis at pH 7.1 ± 0.1

1. NaOH Reservoir.
2. Pump activated by titrator to meter in NaOH.
3. Bacteriological air filter.
4. Inoculation vessel.
5. Autoclavable pH electrode.
6. Sampling port.
7. Sterile sampling syringe and needle.
8. Thermometer well.
9. Baffle.
10. Magnetic drive impeller.
11. Fermentation flask.
12. Water bath maintained at 37°C.

growth during mutan production. The antibiotics were shown to be free of dextranase activity (Section 2.15.7).

Sucrose (BDH Chemicals Limited - Aristar grade) was added to a final concentration of 5% (w/v) and the solution incubated at 37°C for 24h. The mutan was then precipitated by addition of 1 vol. 10% (w/v) sodium acetate solution followed by 2·5 vol. 96% (v/v) ethanol. It was mixed thoroughly, the suspension left to stand at room temperature for 4h and then centrifuged at 1600g for 10 minutes. The precipitate was washed three times with 4·5 vol. of a mixture containing water - 10% sodium acetate - 96% ethanol (2 : 2 : 5 by volume). It was then taken up in 5 - 10 ml of water and lyophilised.

2.9.2 S.glucan was prepared from the mycelium of Aspergillus nidulans strain Eidam biA$_1$ according to the method of Zonneveld (1971).

2.9.3 Dextrans I, II and III were prepared from supernatant samples of cultures of S.sanguis grown in the fermentation cell (Section 2.8) at pH 7·1 $\pm$ 0·1. The culture was harvested during phases I, II and III (Section 3.3) and centrifuged at 1600g for 20 minutes. Dextran was prepared from each supernatant as described for mutan 2 production (Section 2.9.1).

All glucan samples were weighed on a CI Microforce balance MK. IIc. (CI Electronics Limited, Salisbury, Wilts.) and transferred to a dessicator containing P_2O_5 which was maintained at 40°C by a heating mantle. They were dried to constant weight in vacuo.

2.10 CHARACTERISATION OF DEXTRAN - CONTAINING PRECIPITATES

Characterisation of the precipitates obtained in Section 2.9.3 was carried out by means of gas-liquid chromatography, analysis of acidic hydrolysates for glucose and a specific agglutination technique (Gibbons and Fitzgerald 1969).

2.10.1 Gas liquid chromatography. The technique used was a modification of the methods of Lehnhardt and Winzler (1968) and Gheorgiu and Datte (1970).

Dextran (0·14 mg) in a tube (8·5 x 1 cm) fitted with a teflon coated rubber septum, was suspended in 0·1M HCl (0·2 ml). Dowex 50x 2 (H^+ form) (25 mg) was added and hydrolysis carried out at 100°C for 40h. The hydrolysates were neutralised by passing through a column (1·0 x 0·5 cm) of Dowex 1 x 2 (HCO_3^- form, freshly regenerated) and washing through with water (2 x 2·5 ml portions) followed by methanol (2 x 2·5 ml portions). The eluates were pooled, evaporated to dryness and the dry residues reacted with TRI-SIL (Pierce Chemical Company, Rockford, Illinois, 61105, U.S.A.) (100 μl) for 30 minutes. Samples (2 μl) were chromatographed on 12% (w/w) HI-EFF-2BP (ethylene glycol succinate) on gas-chrom P (Applied Science Laboratories, State College, Pennsylvania 16801, U.S.A.) in a coiled glass column (2·1m x 6·35 mm) at 150°C with oxygen free nitrogen as carrier (flow rate 50 ml/min). A Pye 104 Gas Chromatograph with flame ionisation detector was employed.

2.10.2 Hydrolysis of glucans. Glucan samples were weighed on the C.I. Microforce Balance and dissolved in 2M H_2SO_4 to a final concentration of 0·5 - 0·6 mg/ml. They were heated in a glycerol bath at 100°C for 150 minutes, cooled in ice and assayed for glucose (Section 2.15.5) and total carbohydrate (Section 2.15.4). The glucose was expressed as a percentage of total carbohydrate.

2.10.3 Specific agglutination technique. S.mutans OMZ 176 was grown in nutrient broth at 37°C harvested by centrifugation at 1600g for 20 min, washed in 0·9% (w/v) sodium chloride and suspended in 0·067M sodium phosphate buffer, pH 8·0, to a concentration of approximately 10^9 cells/ml. Dextran was dissolved in the same buffer to give a concentration of 100 μg/ml. Dextran solution (0·2ml) was mixed with cell suspension (0·3ml) and incubated at 35°C. Controls from which cells or dextran were omitted were also studied in order to eliminate the possibility of auto-agglutination. The tubes were examined visually and by light microscopy at regular intervals

up to 72h for signs of agglutination. Samples of dextran-treated cells of S. mutans and untreated control cells were diluted 10,000 fold with 0·067M sodium phosphate buffer, pH 8·0, mixing carefully in order to minimise mechanical breakdown of bacterial clumps. Drops of this suspension were placed on Maxtaform electron microscope grids, type H9 (Taab Laboratories, Kidmore End Road, Emmer Green, Reading, England), the excess liquid blotted off and the grids washed with water, dried and examined under the electron microscope (Model AE1 EM 6B).

2.11 STRUCTURAL EXAMINATION OF DEXTRANS

2.11.1 Infra-red spectroscopy. Prepared glucan samples (Section 2.9) were pressed into KBr discs and infra-red spectra prepared from them in a Pye 225 spectrophotometer.

The spectra were examined for the presence of peaks characteristic of α- and β- glucopyranose ring structure and the different glycosidic linkage types. Comparisons were drawn between the spectra of glucans of known and unknown structure (Barker et al, 1957).

2.11.2 Enzymic degradation. The technique used was that described by Newbrun (1972). The increase in reducing power with time was measured by the Somogyi method (Section 2.15.3), using maltose as a standard. The maltose production after 3h was taken as a measure of the relative susceptibility of the glucan to the enzyme.

2.11.3 Periodate oxidation. 20ml standardised sodium meta-periodate solution (approximately 0·01M – Section 2.15.8) was added to 40mg dextran and stirred in the dark at 4°C. Aliquots were removed at known time intervals and assayed for periodate (Section 2.15.8). The percentage α-(1→6)- linkages and/or end groups were then calculated from the amount of periodate consumed.

2.12 PARTIAL PURIFICATION OF DEXTRANSUCRASE

Enzyme production was carried out as in Section 2.8 and, at the desired stage, culture fluid was aseptically siphoned into a flask cooled to 4°C. The cell suspension was then pumped into a continuous action rotor (Measuring and Scientific Equipment Limited, Buckingham Gate, London, S.W.1) at 150 ml/min and centrifuged at 15,000g at 4°C and the supernatant collected in an ice-cooled flask. Antibiotics (100µg/ml) were added to prevent growth of any bacteria left in suspension.

2.12.1 Hydroxylapatite chromatography.

(i) Batch-wise elution (Carlsson, 1969). A slurry of hydroxylapapite in 0·001M phosphate buffer, pH 6·8 as supplied by Sigma Biochemicals Ltd., was added to the supernatant (100 - 200 ml slurry/litre supernatant) and stirred overnight at 4°C. It was then centrifuged down and washed with 0·05M potassium phosphate buffer, pH 6·0 at 4°C for 4h with stirring. It was centrifuged at 1600g for 10 minutes and washed successively with 0·1M, 0·15M and 0·2M potassium phosphate buffers pH 6·0. It was finally eluted with 0·5M potassium phosphate buffer, pH 6·8. At each step the volume of washings was measured and a sample assayed for dextransucrase (Section 2.15.2ii) and protein content (Section 2.15.6i). Active samples were pooled and concentrated (Section 2.13).

(ii) Column chromatography. The culture supernatant was adsorbed onto hydroxylapatite as described above and washed with potassium phosphate buffers pH 6·0 up to 0·2M potassium phosphate buffer pH 6·0 as described above. 250 ml of the precipitate was then packed to a depth of 20 - 40 m m into a 63 m m bore chromatography column with long adjustable column ends (Jobling Laboratory Division, Stone, Staffs.) according to the makers instructions. 0·2M potassium phosphate buffer pH 6·0 was pumped through by means of a chromapump

at 14·4 ml/h (Baird and Tatlock Limited, Blackhorse Lane, Walthamstow, London) until the column had packed down.

The column was eluted by means of a linear gradient set up between 0·2M potassium phosphate buffer, pH 6·0 (750 ml) and 1·0M phosphate buffer pH 6·8 (750 ml). The flow rate was 14·4 ml/h and 3·6 ml fractions were collected by means of a chromafrac fraction collector (BTL). The apparatus was set up in a cold room at 4°C. Every third sample was assayed for protein (Section 2.15.6ii) and dextransucrase (Section 2.15.2ii) and subsequently all fractions around the peaks were assayed.

2.12.2 Ultrafiltration and Concentration.

The elution pattern obtained from hydroxylapatite chromatography was examined and suitable active samples pooled as indicated in the results (Section 3.6.1.ii). Desalting and concentration of pooled fractions was then carried out using an ultrafiltration cell or hollow fibre device (Section 2.13).

2.13 DIALYSIS AND CONCENTRATION OF MATERIAL

2.13.1 Dialysis.

Visking tubing (Scientific Instrument Centre, Leeke Street, London) was cut into desired lengths, suspended in 1 l distilled water and heated at 60°C in a water bath for 2 hours. The water was discarded and the process repeated twice more. It was then washed at room temperature three times with 98% ethanol, and three times with distilled water. It was then stored in distilled water at 4°C until required. Dialysis was carried out against distilled water at 4°C with stirring.

2.13.2 Ultrafiltration and Concentration.

i) Hollow fibre device. Rapid dialysis and/or concentration of solutions was achieved with a Bio-Fiber Beaker (Bio-Rad Laboratories Ltd., Bromley, Kent). The sample (100 ml) and a magnetic stirring bar were placed in the beaker surrounding

the fibre bundle. To desalt the solution, distilled water was run through the fibre bundle from a gravity feed at approximately 100 ml/min for 30 minutes.

To concentrate and desalt the solution, distilled water was pumped into the fibre bundle at 40 ml/h and removed by a second pump at 90 ml/h. It was run overnight or until the sample had been sufficiently concentrated.

The apparatus was set up in a cold room at 4°C with stirring.

ii) Ultrafiltration cell. Amicon ultrafiltration cells (Amicon Corporation, Lexington, Massachusetts 02173) with Diaflo PM 10 ultrafilters (Amicon) were used to concentrate solutions by applying positive pressure (414 kPa).

2.14 PROPERTIES OF DEXTRANSUCRASE

2.14.1 Optimum pH (Carlsson et al, 1969). Aristar sucrose was dissolved in the following buffers to a final concentration of 10% (w/v) and each buffer was supplemented with penicillin/streptomycin mixture to give a final concentration in the assay incubation mixture of 100 µg/ml. The buffers used were 0·1M sodium phosphate - citrate buffer (Dawson et al, 1962) for pH values of 3·6, 4·0, 4·6, 5·0, 5·6, 6·0 and 6·4, 0·1M sodium phosphate buffer for values of 6·4, 6·8, 7·0 and 7·2, 0·1M veronal buffer for values 7·2, 7·6 and 8·0 and 0·1M sodium borate buffer for pH 8·0, 8·4 and 9·0. Partially purified enzyme (0·5ml) was then added to 0·5 ml of each buffered sucrose solution and the pH was re-measured. The tubes were then incubated at 37°C for 6 hours. The assay for dextransucrase was then continued as in Section 2.15.2 ii. Assays were performed in duplicate.

2.14.2 Stability. The stability of dextransucrase under different conditions of storage was examined. Samples of crude supernatant (Section 2.8) were stored at room temperature at 4°C and at -20°C for known periods of time. Samples which had been frozen and thawed several

times in succession were also studied. The effect of these conditions on dextransucrase activity was examined.

2.15 ASSAYS

2.15.1 Cell Growth. Bacterial cell density was measured by determining the turbidity of the culture at 540 nm. A culture of S.sanguis in Carlsson's diffusate medium was grown at 37°C without pH control and samples of 200 ml were removed at intervals. The optical density was measured immediately at 540 nm and the sample was then centrifuged at 1600 g for 20 minutes. The supernatant was discarded and the cells washed twice with 200 ml 0·1M sodium phospate buffer, pH 7·0 and twice with 100 ml distilled water. The washings were discarded and the cells dried over P_2O_5 in vacuo until they reached constant weight. The correlation was then shown between the optical density of the culture sample at 540 nm and its dry weight.

2.15.2 Dextransucrase.

i) Carlsson's method (1969). An equal volume of 0·25M sucrose in 0·1M sodium phosphate buffer pH 6·8 was added to a sample of culture supernatant at 37°C 0·2 ml samples were removed at zero time and at known time intervals and added immediately to 0·8 ml 0·04 M NaOH. The samples were then assayed for fructose by the Somogyi method (Section 2.15.3).

ii) Cybulska and Pakula's method (1963). One volume 10% (w/v) sucrose in 0·1M sodium phosphate buffer, pH 7·0, containing streptomycin/penicillin (200µg/ml) was added to one volume of culture supernatant. It was mixed well and incubated at 37°C for 24 hours. Two volumes 10% (w/v) anhydrous sodium acetate solution followed by 5 vol. 96% ethanol were added

and mixed well to precipitate the dextran. The suspension was left to stand at room temperature for 4 hours and the precipitate centrifuged down at 1600g for 20 minutes. It was washed twice with a mixture containing water - 10% (w/v) sodium acetate - 96% ethanol (2 : 2 : 5 by volume) and left to drain. The final precipitate was dissolved in 0·1M NaOH and the dextran determined by the phenol - H_2SO_4 method (section 2.15.4).

2.15.3 Reducing Sugar. This was determined by the method of Somogyi (1945). Due to problems of surface oxidation, it was necessary to include fructose standards of 0·1 to 1·0 mg/ml fructose with every batch of assays.

2.15.4 Total Carbohydrate. The phenol - H_2SO_4 method was employed (Dubois et al, 1956). All assays were performed in triplicate and glucose standards included. A standard curve was prepared using 10 - 100µg/ml glucose.

2.15.5 Glucose. The GOD-Perid method for blood sugar was carried out using a Biochemica Test Combination (Boehringer Corporation Limited, Uxbridge Road, Ealing, London).

2.15.6 Protein.

i) Lowry method. The method of Lowry et al (1951) was used with bovine α-chymotrypsinogen standardised by its extinction coefficient as a standard (Wilcox et al, 1957).

ii) U.V. Absorbance. The protein concentration of column eluates was measured by the absorbance of u.v. light at 280 nm using cuvettes of 1 cm light path in a SP.500 spectrophotometer (Unicam Instruments Limited, York Street, Cambridge, England) and a standard of bovine α-chymotrypsinogen.

2.15.7 Dextranase. Antibiotic sample (1 ml) (300 μg/ml) was added to 2 ml 1·67% (w/v) dextran 2000T in 0·1M sodium phosphate buffer, pH 7·0 as described in the Worthington Manual (1972). It was incubated at 37°C. and at known time intervals, 0·2 ml aliquots were added to 0·8 ml 0·04M NaOH and assayed for reducing sugar by the Somogyi method (section 2.15.3). Dextranase (Worthington) 0·4 μg/ml was assayed by the same method for purposes of comparison.

2.15.8 Periodate. 0·02M standard arsenic trioxide solution was prepared and used to standardise an approximately 0·01M iodine solution according to the method of Dyer (1956). Subsequently solutions of sodium meta-periodate could be titrated against the standardised iodine and the periodate content determined.

3. RESULTS

3.1 Growth of S.sanguis.

3.1.1 Rate of growth and pH change of S.sanguis cultures.

(i) The turbidity of the culture in relation to the dry weight. A direct relationship was shown between the turbidity of a culture sample at 540 nm and the dry weight of that sample, up to an extinction value of 1·4 (Fig.3.1). The cells had been washed twice with 0·1M sodium phosphate buffer, pH 7·0, to remove the medium in which they had been grown and then with distilled water to remove all salts, before drying to constant weight. Without this procedure, salts adhering to the dried cells would add to the weight, giving erroneous results. However, it is possible that some cells were lysed during the washing with distilled water, although this step was carried out rapidly to keep lysis to a minimum.

(ii) Rate of growth of the culture. After an initial lag phase of 4-5h a rapid increase in cell density followed, reaching a maximum within 3h (Fig.3.2). During this rapid growth phase, the doubling time was approximately 1h. The turbidity then declined slowly.

(iii) pH change of the culture. The pH of sterile Carlsson's diffusate medium was 7·0 - 7·3. Inoculation with S.sanguis caused a slight drop in pH (to 6·8 - 7·1) due to the small amount of acid introduced with the inoculation from the starter culture. This pH value remained constant until the cell density started to increase and, as the cell density rapidly rose, the pH fell to a value of 4·4 - 4·8 (Fig.3.2). The pH remained constant at this value.

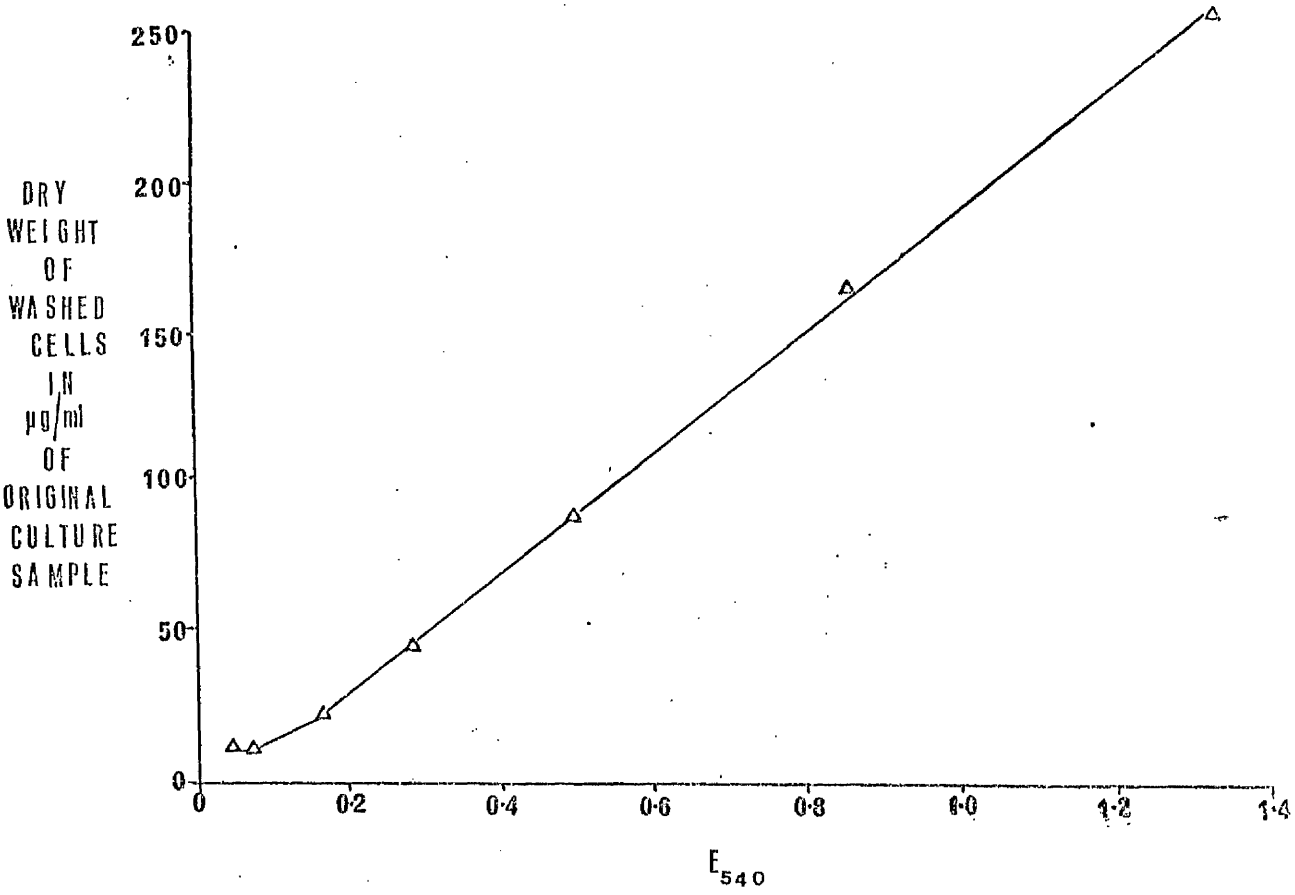


FIG. 3.1 THE RELATIONSHIP BETWEEN EXTINCTION AT 540 nm AND THE DRY WEIGHT OF THE CELLS/ml IN S. sanguis CULTURE SAMPLES.

The extinction of culture samples of S. sanguis grown in Carlsson's diffusate medium at 37°C, with stirring, was measured at 540 nm. 200 ml samples were then centrifuged at 1600g for 20 mins to remove the cells which were then washed twice with 200 ml 0·1M sodium phosphate buffer, pH 7·0 and twice with 200 ml distilled water. They were then dried to constant weight over P_2O_5 in vacuo.

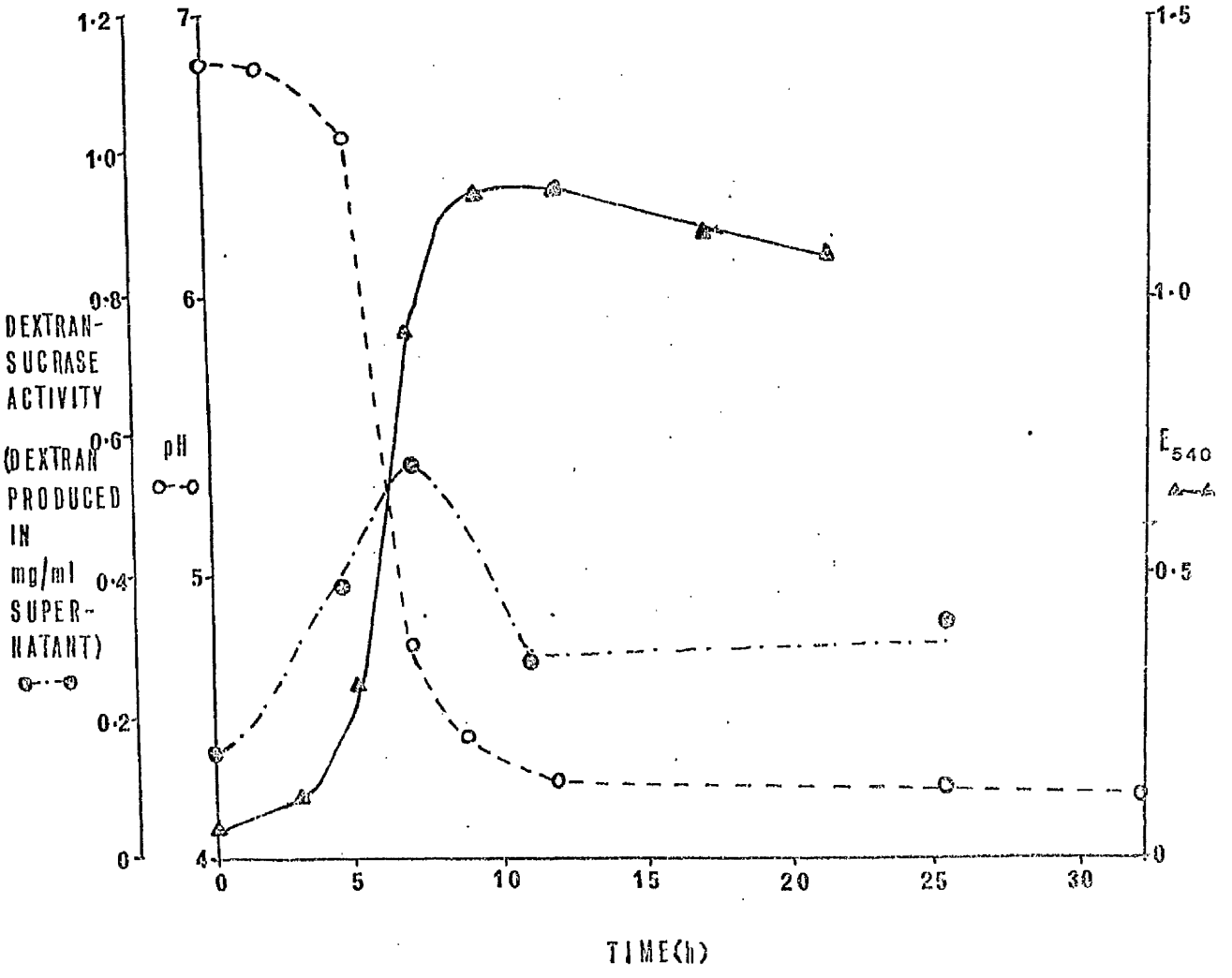


FIG.3.2 GROWTH OF AND DEXTRANSUCRASE PRODUCTION BY S.sanguis.

200 ml Carlsson's diffusate medium was inoculated with a few drops of a culture of S.sanguis and incubated at 37°C with shaking. The cell density (▲—▲), pH (o - - -o) and dextransucrase activity (●-·-●) were measured as described in Section 2.15.2ii.

3.1.2 Dextransucrase production by S.sanguis.

(i) Choice of dextransucrase assay. Time course experiments using Carlsson's (1969) dextransucrase assay and crude supernatant samples revealed an initial drop in reducing power after ten minutes (Fig.3.3) followed by an increase in reducing groups for a further twenty minutes and finally the reducing power declined over the next 30 - 50 minutes.

However, enzyme samples which had been partially purified by hydroxylapatite chromatography as described in Section 2.12.ii gave an approximately linear reaction rate over 60 mins (Fig.3.4).

Cybulska and Pakula's (1963) method was based upon the assay of precipitable carbohydrate by the anthrone method. The phenol/H_2SO_4 method for the assay of total hexose was considered to be more accurate and so the method of Cybulska and Pakula was modified to include the phenol/H_2SO_4 method instead of the anthrone method.

Time course experiments using the modified Cybulska and Pakula method gave an approximately linear reaction rate over 24h (Fig.3.5) and this method was, therefore, adopted for assay of crude supernatant samples for dextransucrase. Carlsson's method was considered to be unsuitable for the assay of crude supernatant samples as changes in other reducing substances in the supernatant appeared to be interfering with the measurement of reducing sugars in the assay.

(ii) The use of antibiotics in Cybulska and Pakula's modified dextransucrase assay. Cybulska and Pakula's modified dextransucrase assay involves incubation of supernatant samples at

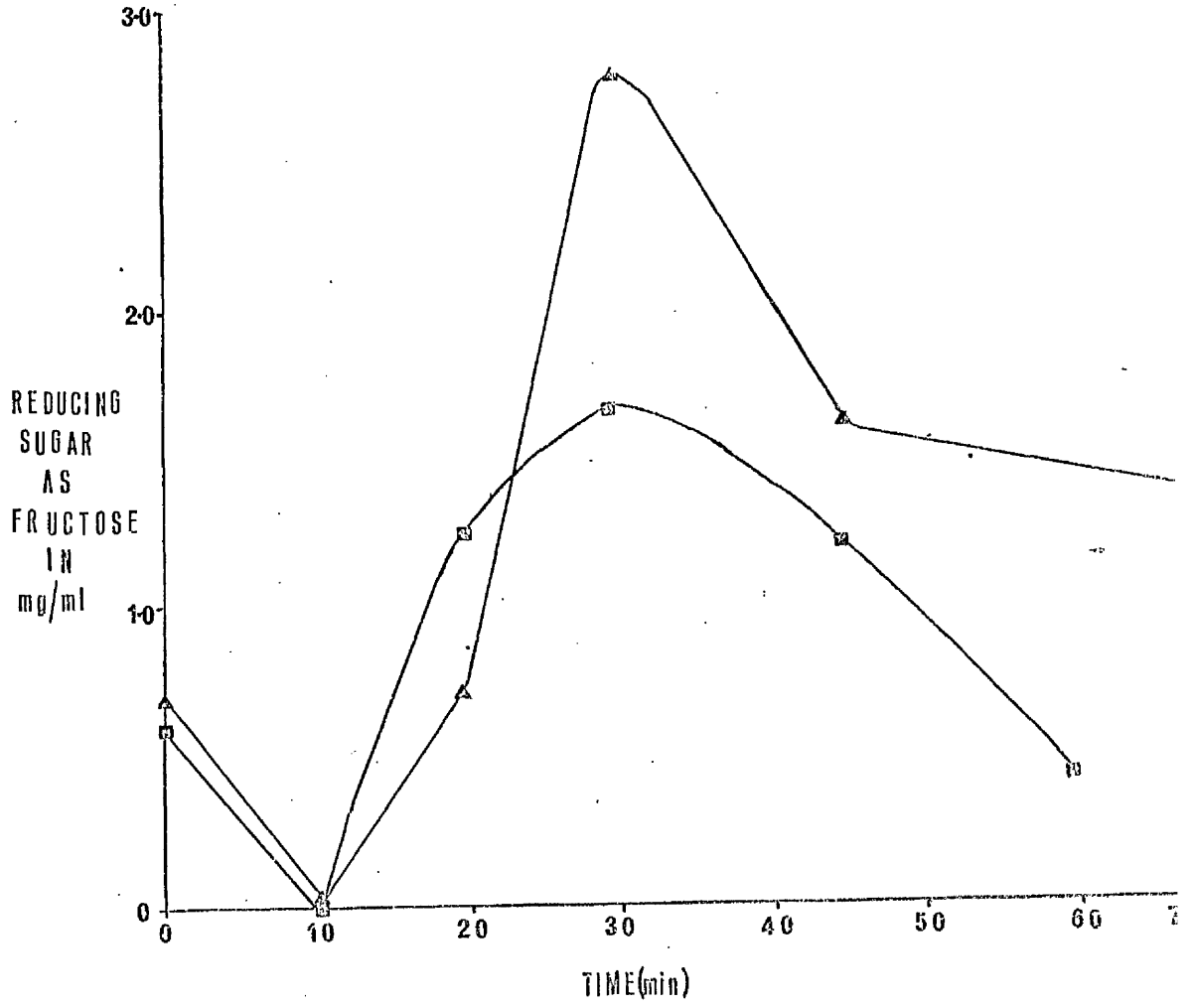


FIG.3.3 THE TIME COURSE FOR DEXTRANSUCRASE ASSAY (1) AS DESCRIBED BY CARLSSON (1969).

The assay mixture contained 1 ml culture supernatant and 1 ml 0·25M sucrose in 0·1M sodium phosphate buffer, pH 6·8. It was incubated at 37°C and 0·2 ml samples were removed at zero time and at regular intervals up to 70 mins. The samples were assayed immediately for reducing sugar by the Somogyi method. The reducing sugar liberated with time for two different crude supernatant samples of different enzyme activities is shown.

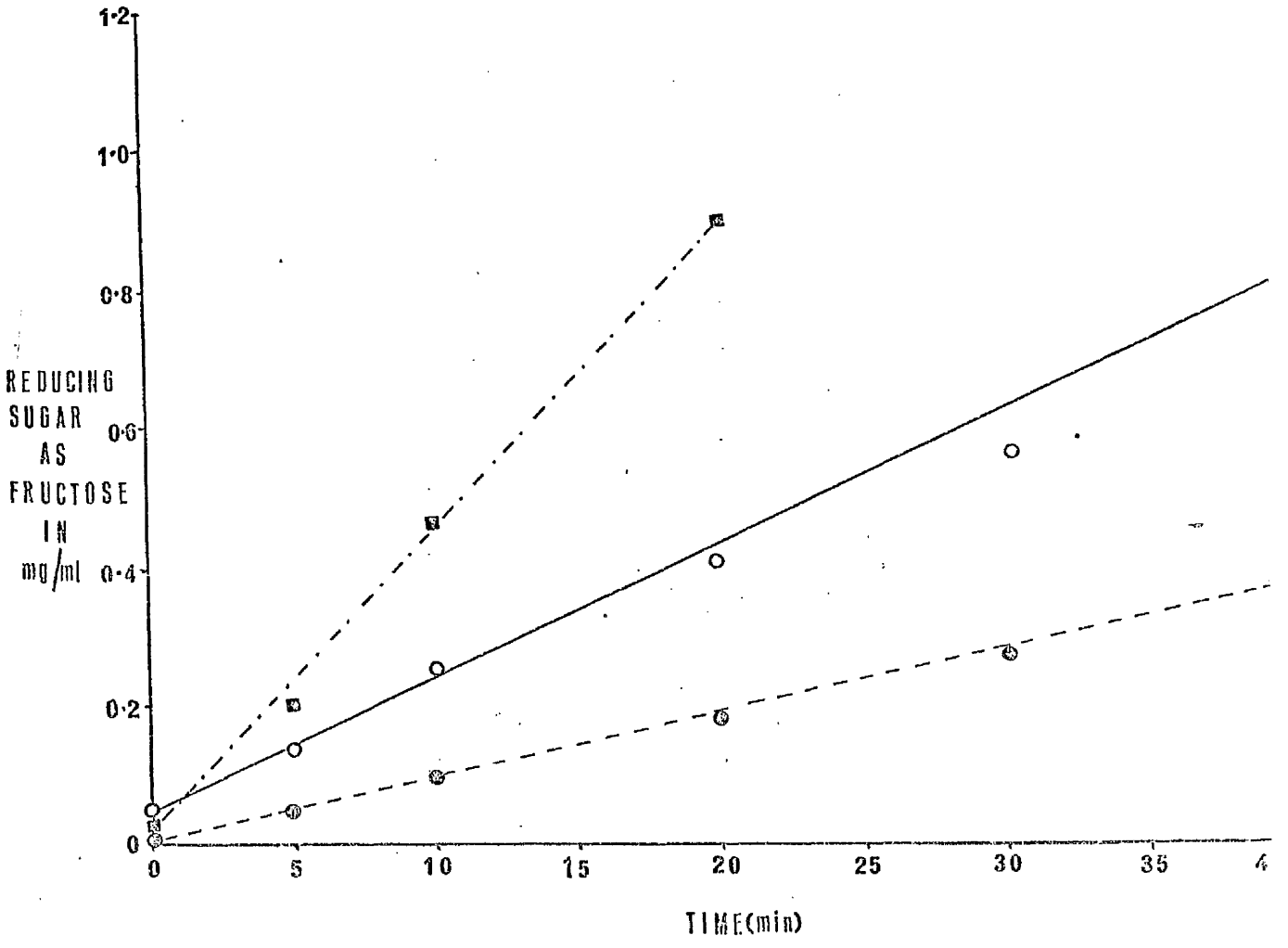


FIG.3.4 THE TIME COURSE FOR DEXTRANSUCRASE ASSAY (2) AS DESCRIBED BY CARLSSON (1969).

The assay was carried out as for Fig.3.3. Enzyme samples which had been partially purified by hydroxylapatite chromatography as in Section 2.12.1i were used instead of culture supernatant. These samples were the washings obtained from 0·2M phosphate buffer, pH 6·0, which had been desalted (Section 2.13.2i) and diluted in the assay buffer. The reducing sugar liberated with time for three enzyme samples of different enzyme activities is shown.

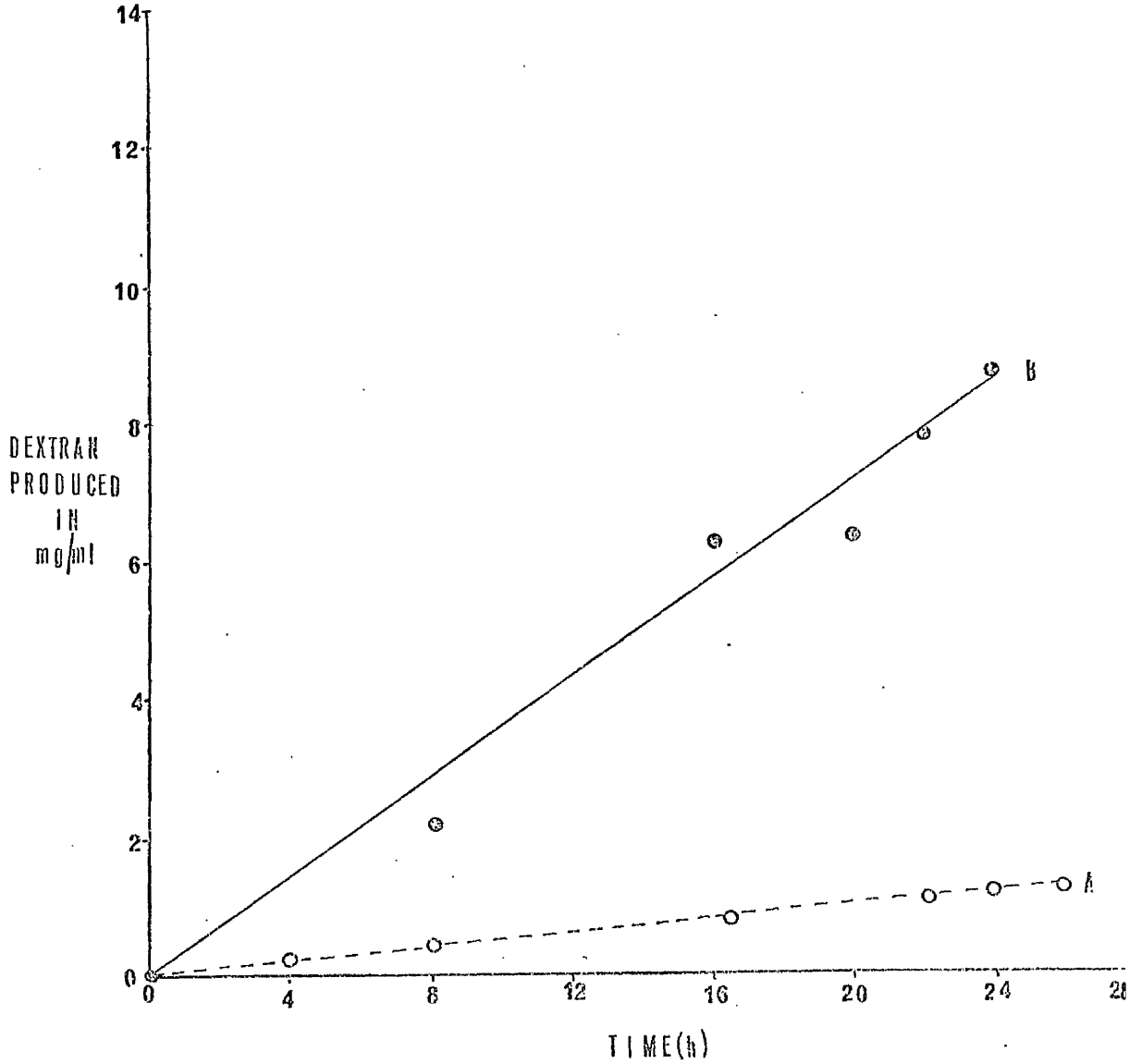


FIG.3.5 THE TIME COURSE FOR A MODIFIED METHOD OF THE DEXTRANSUCRASE ASSAY DESCRIBED BY CYBULSKA AND PAKULA (1963)

The assay was carried out as described in Section 2.15.2ii. The dextran produced with time for one crude supernatant sample (A) and one sample (B) which had been partially purified by hydroxylapatite chromatography as described in Section 2.12.1i is shown.

37°C for 24h in the presence of sucrose - see Section 2.15. 2ii. These conditions would favour growth of any bacteria left in the supernatant or entering from the atmosphere and such growth would interfere with the assay. The addition of penicillin/streptomycin to the assay mixture prevented bacterial growth during the 24h incubation period.

The antibiotics were of fungal origin but some fungi produce dextranase (Ceska et al, 1972) and it was, therefore, necessary to ensure that no active dextranase was present in the antibiotics to interfere with the assay.

The antibiotics were shown to be free of dextranase activity (Fig. 3.6) and the assay system used was shown to be capable of detecting dextranase activity by means of a commercial dextranase preparation.

(iii) Dextransucrase production pattern. The change in dextransucrase activity of a culture of S. sanguis grown without pH control is shown in Fig. 3.2 . Dextransucrase activity reached a peak when the growth of the culture was at a maximum. It then declined rapidly as the growth ceased and the pH fell below approximately 5·0 (Fig. 3.2). Production of the enzyme stopped when growth had stopped and the pH had reached its lowest level.

3.1.3 Conclusions. The measurement of the extinction of culture samples at 540 nm. is a valid method of obtaining a measure of bacterial mass.

The modified Cybulska and Pakula method is a more suitable method of measuring the dextransucrase activity of culture supernatant samples than Carlsson's method.

The addition of penicillin/streptomycin to supernatant samples prevents bacterial growth but does not interfere with

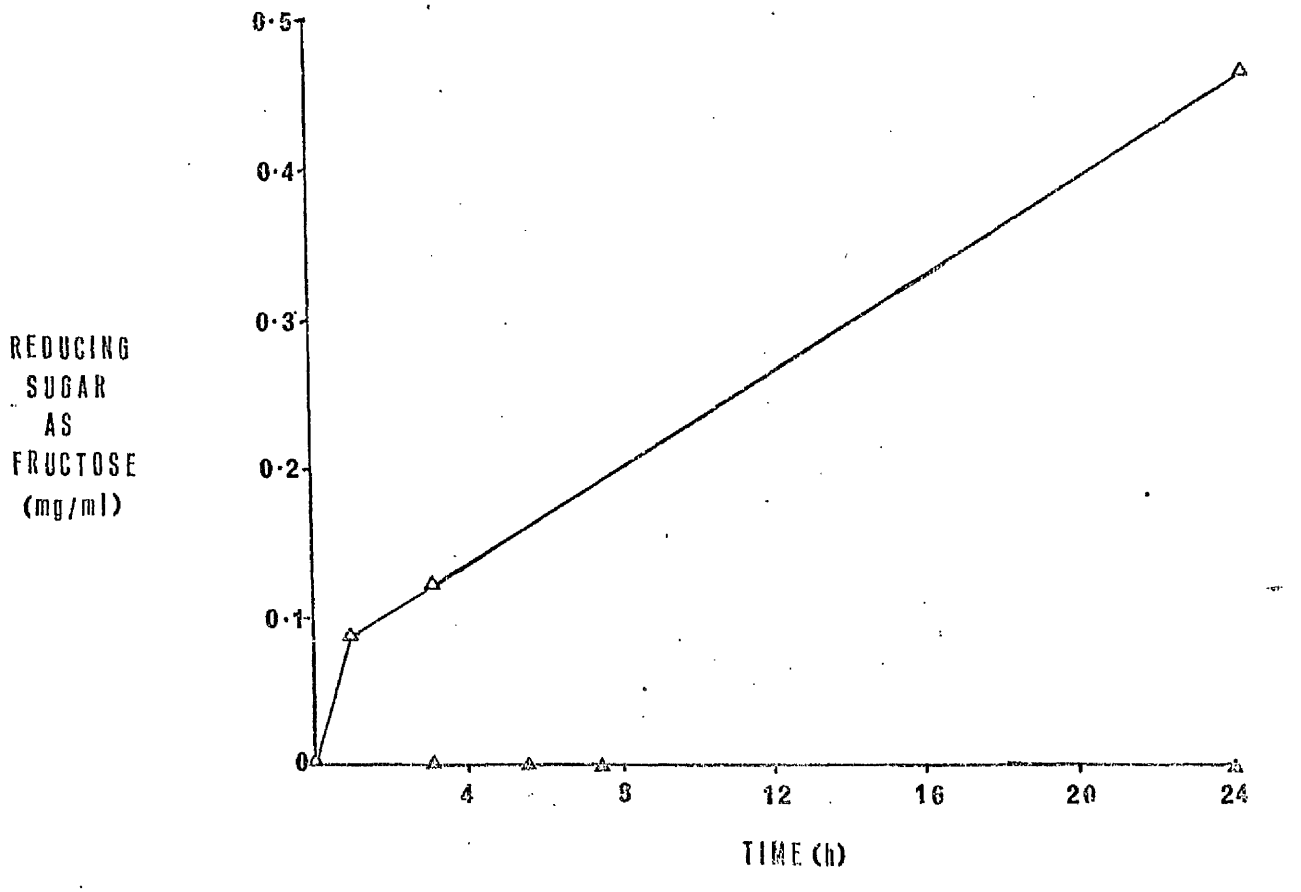


FIG.3.6 DEXTRANASE ASSAY ON PENICILLIN/STREPTOMYCIN ANTIBIOTIC MIXTURE

The assay mixture contained 1 ml antibiotic sample (300µg/ml.) and 2 ml. 1·67% (w/v) dextran T2000 in 0·1M sodium phosphate buffer, pH 7·0, incubated at 37°C. 0·2 ml samples were removed at zero time and known time intervals and assayed for reducing sugar by the Somogyi method (Δ—Δ). To ensure the validity of the assay procedure, 1 ml dextranase (0·4µg/ml.) was added to 2 ml. 1·67% (w/v) dextran T2000 in 0·1M sodium phosphate buffer, pH 7·0 and assayed as above (Δ---Δ).

dextransucrase assays.

S.sanguis grows rapidly at 37°C in Carlsson's diffusate medium producing copious amounts of acid which leads to a very rapid decrease in pH. Growth ceases when the pH falls below approximately 5·0. Cessation of growth may be due to an unfavourable pH, overcrowding of bacteria, the accumulation of noxious substances, removal of necessary nutrients or a combination of any two or more of these factors.

Similarly, dextransucrase activity reaches a peak when growth is maximal, but rapidly declines when the pH falls below approximately 5·0 and growth ceases.

This drop in activity may be due to instability of the enzyme at this low pH as the activity is rapidly lost with the fall in pH (see Section 3.7.1).

Acid production may limit growth and dextransucrase production. Neutralisation of the acid as it is produced may remove this limiting factor and allow growth and dextransucrase activity to reach greater levels.

3.2 DEVELOPMENT OF OPTIMUM CONDITIONS FOR DEXTRANSUCRASE PRODUCTION

3.2.1 The effect of inoculum size. Growth of cultures inoculated with a small inoculum (0·5% 16h culture) was as rapid as cultures with a larger inoculum, but there was a longer lag phase before growth started and the final bacterial yield was slightly lower (Fig.3.7,0·5% and 2·5% 16h culture). There was little difference in the dextransucrase activity observed (Fig.3.8), similar yields being produced at approximately the same time after inoculation with a 5 ml 16h inoculum as with 1 ml of the same starter culture.

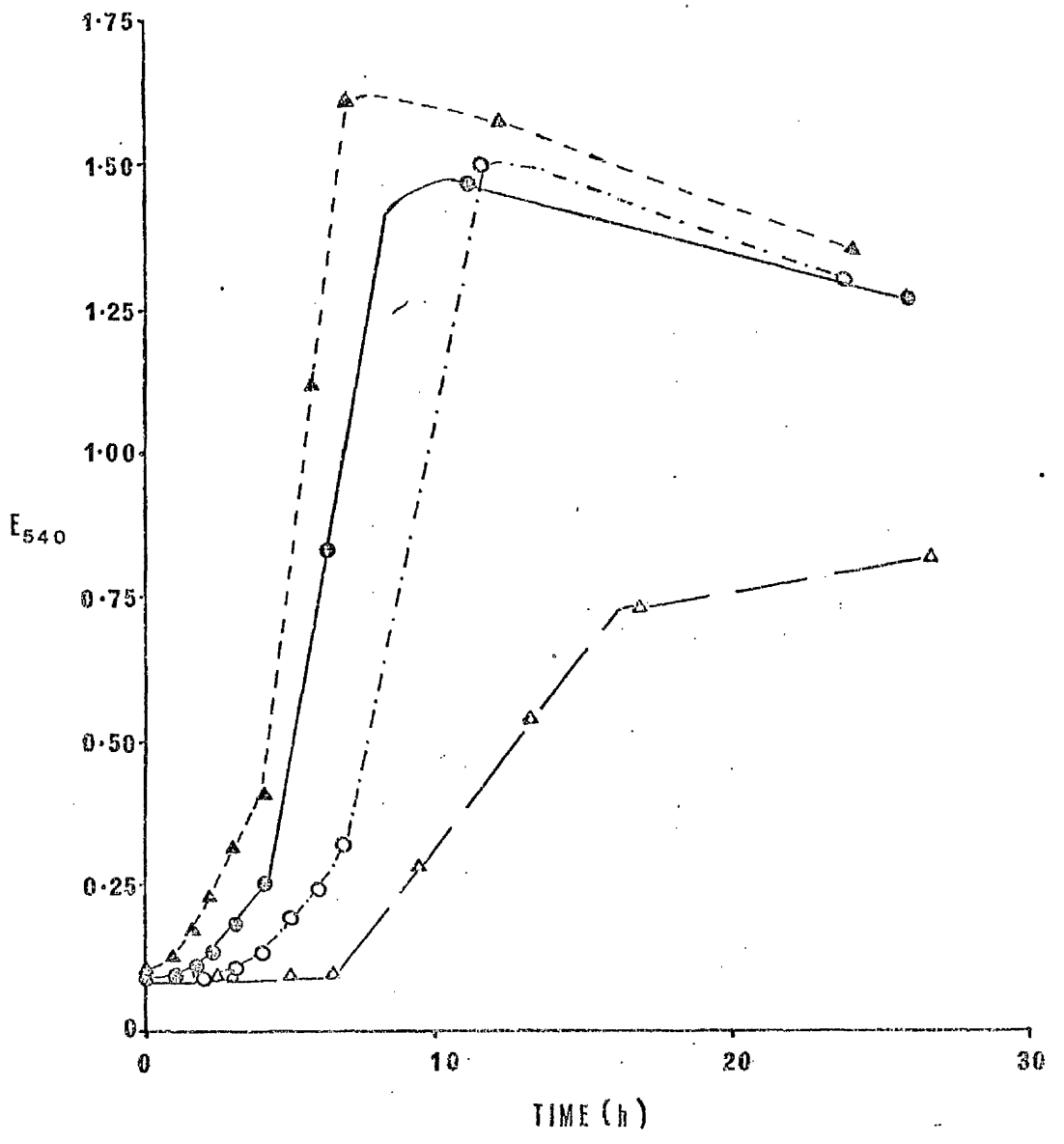


FIG.3.7 EFFECT OF INOCULUM SIZE AND AGE OF INOCULUM CULTURE ON GROWTH OF S.sanguis.

Flasks containing 200 ml Carlsson's diffusate medium were inoculated with 5 ml of a 16h culture of S.sanguis (▲ - - ▲), 1 ml of a 16h culture (●——●), 5 ml of a 7h culture (▲——▲) or 5 ml of a 10½h culture (o - · - o) respectively. The cell density, measured as extinction at 540 nm (E_{540}) was measured at known time intervals.

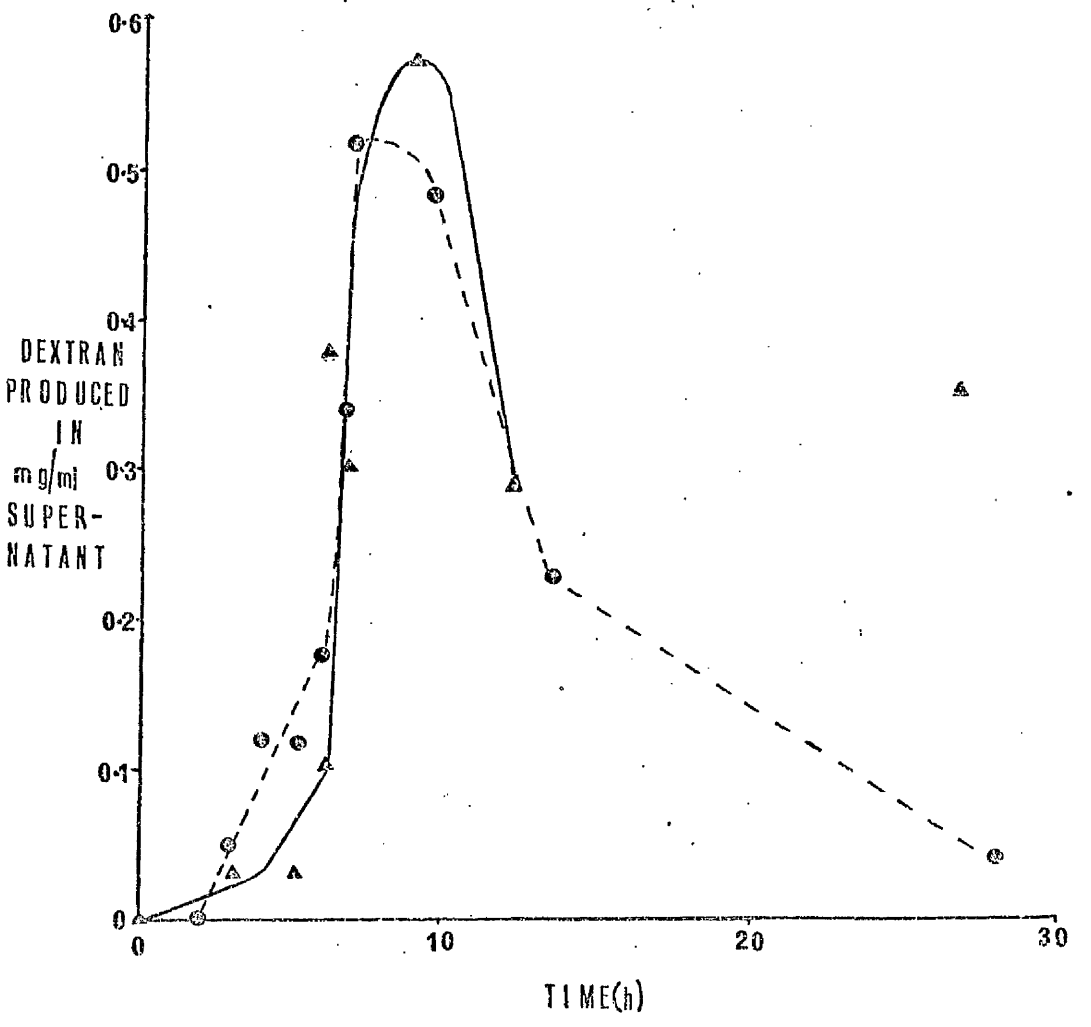


FIG.3.8 THE EFFECT OF INOCULUM SIZE ON DEXTRANSUCRASE PRODUCTION BY S.sanguis.

Flasks containing 200 ml Carlsson's diffusate medium were inoculated with 5 ml of a 16h culture of S.sanguis (▲——▲), or 1 ml of a 16h culture (● - - ●). Aliquots of supernatant were assayed for dextransucrase activity as described in Section 2.15.2ii.

3.2.2 The effect of inoculum age. The age of the culture inoculum had a marked effect on subsequent growth and dextransucrase production. The younger the inoculum, the longer the lag phase of growth and the lower the bacterial yield (Fig.3.9). Dextransucrase activity reached a peak when bacterial yield attained its maximum and consequently dextransucrase production occurred later with younger inocula (Fig.3.9). The yield of dextransucrase was also lower with younger inocula.

3.2.3 The effect of pH control. When S.sanguis was inoculated into Carlsson's diffusate medium maintained at pH 5·1 ± 0·1, no growth occurred. At all other pH values examined (6·1 ± 0·1, 6·6 ± 0·1, 7·1 ± 0·1, 7·6 ± 0·1 and 8·1 ± 0·1) both bacterial yield and dextransucrase production were increased when compared with those of cultures without pH control (Figs.3.10-3.14). At pH 6·1 the doubling time was approximately twice that in cultures without pH control, but the bacterial yield in the culture maintained at pH 6·1 ± 0·1 was 2-3 times greater. At pH 6·6 ± 0·1 and pH 7·1 ± 0·1 the doubling time was the same as in the culture without pH control, but the bacterial yields were more than four times greater. At pH 7·6 ± 0·1 and 8·1 ± 0·1 growth was much slower than at the other pH values. At pH values 6·6 ± 0·1, 7·6 ± 0·1 and 8·1 ± 0·1 the bacteria tended to form aggregates which were difficult to keep in suspension. This interfered with the measurement of the extinction at 540 nm and measurements were, therefore, stopped when aggregation occurred. At pH 6·6 ± 0·1 aggregation occurred at very high cell densities (Fig.3.11) but at pH 7·6 ± 0·1 and 8·1 ± 0·1 the turbidity was approximately 1·5 when aggregation occurred (Figs.3.13-3.14). In those cultures where turbidity could be measured, growth ceased when all the glucose in the medium had been utilised (Fig.3.10-3.12).

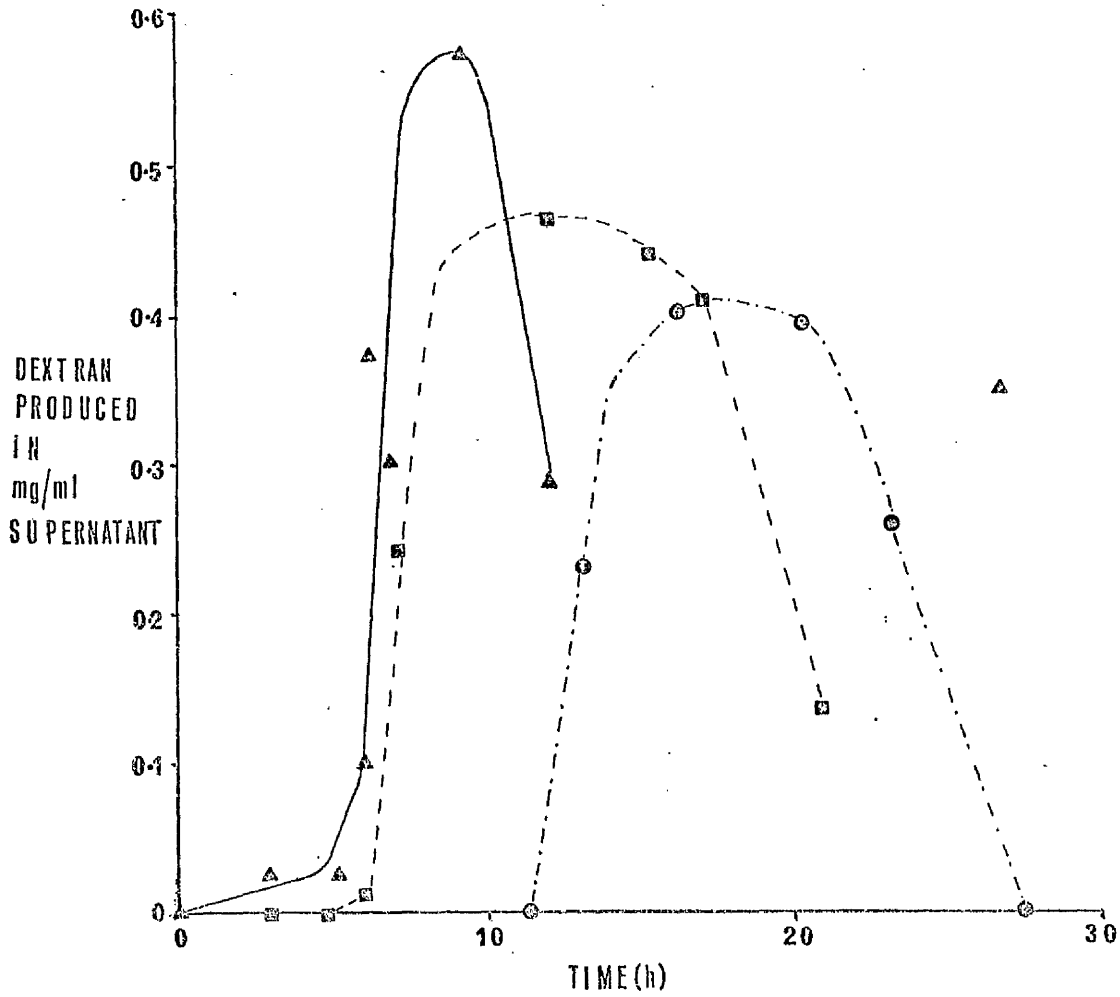


FIG.3.9 THE EFFECT OF INOCULUM CULTURE AGE ON DEXTRANSUCRASE PRODUCTION BY S.sanguis.

Flasks containing 200 ml Carlsson's diffusate medium were inoculated with 5 ml of a 16h culture of S.sanguis (▲——▲), 5 ml of a 10½h culture (▣ - - ▣) or 5 ml of a 7h culture (● - · - ●) respectively. Aliquots of supernatant were assayed for dextransucrase activity as described in Section 2.15.2ii.

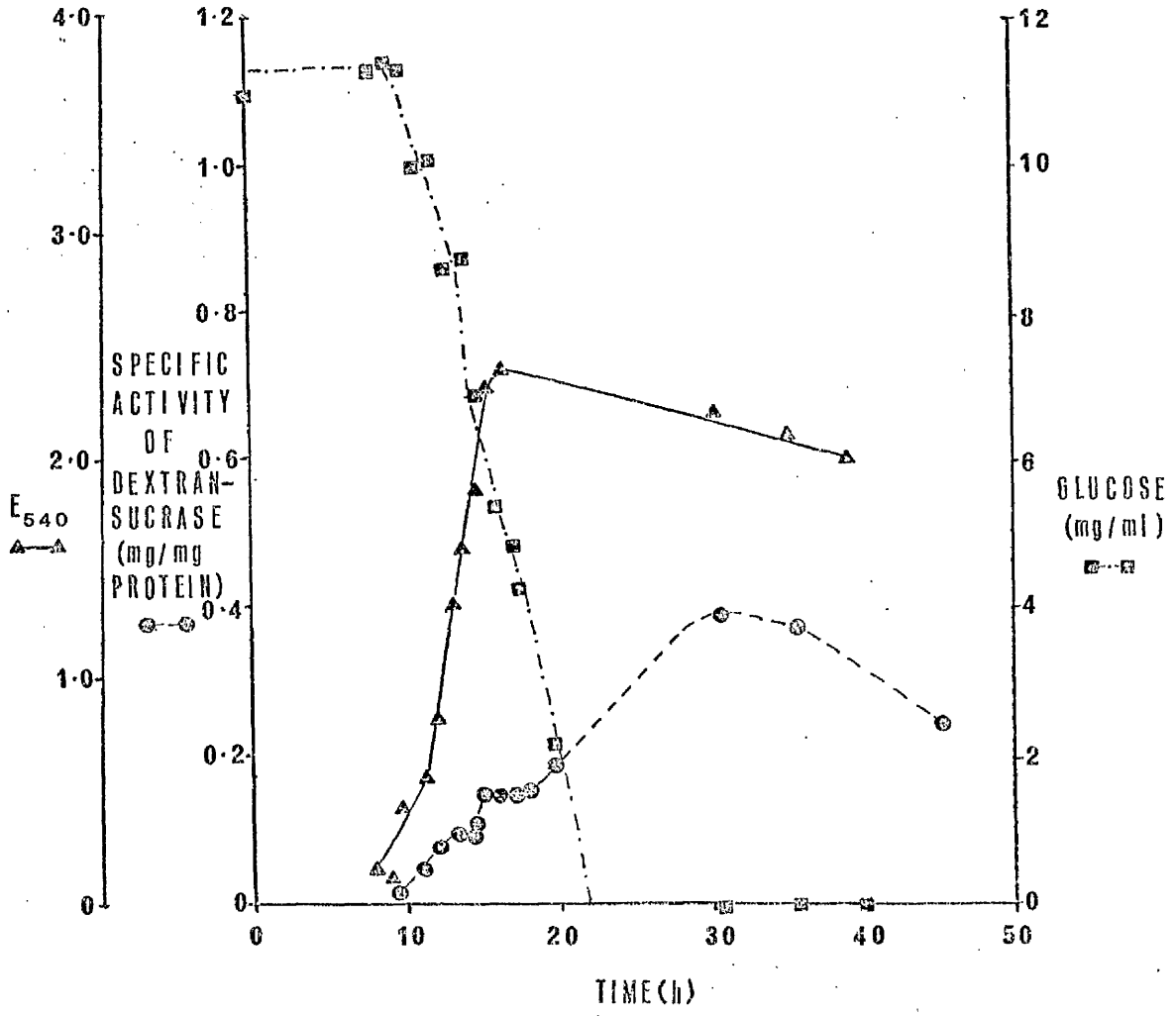


FIG. 3.10 DEXTRANSUCRASE PRODUCTION BY S. sanguis AT pH 6·1 ± 0·1.

200 ml Carlsson's diffusate medium, maintained at pH 6·1 ± 0·1, was inoculated with a 2·5% 16h inoculum of S. sanguis at 37°C, with stirring. Samples were removed at known time intervals and the extinction measured at 540 nm (▲—▲). Aliquots of supernatant were assayed for glucose by the glucose oxidase method (■ -·- ■) and protein by the Lowry method. The specific activity of dextransucrase (● - - ●) is the amount of precipitable carbohydrate produced, as measured by the phenol/H_2SO_4 method, per mg of protein.

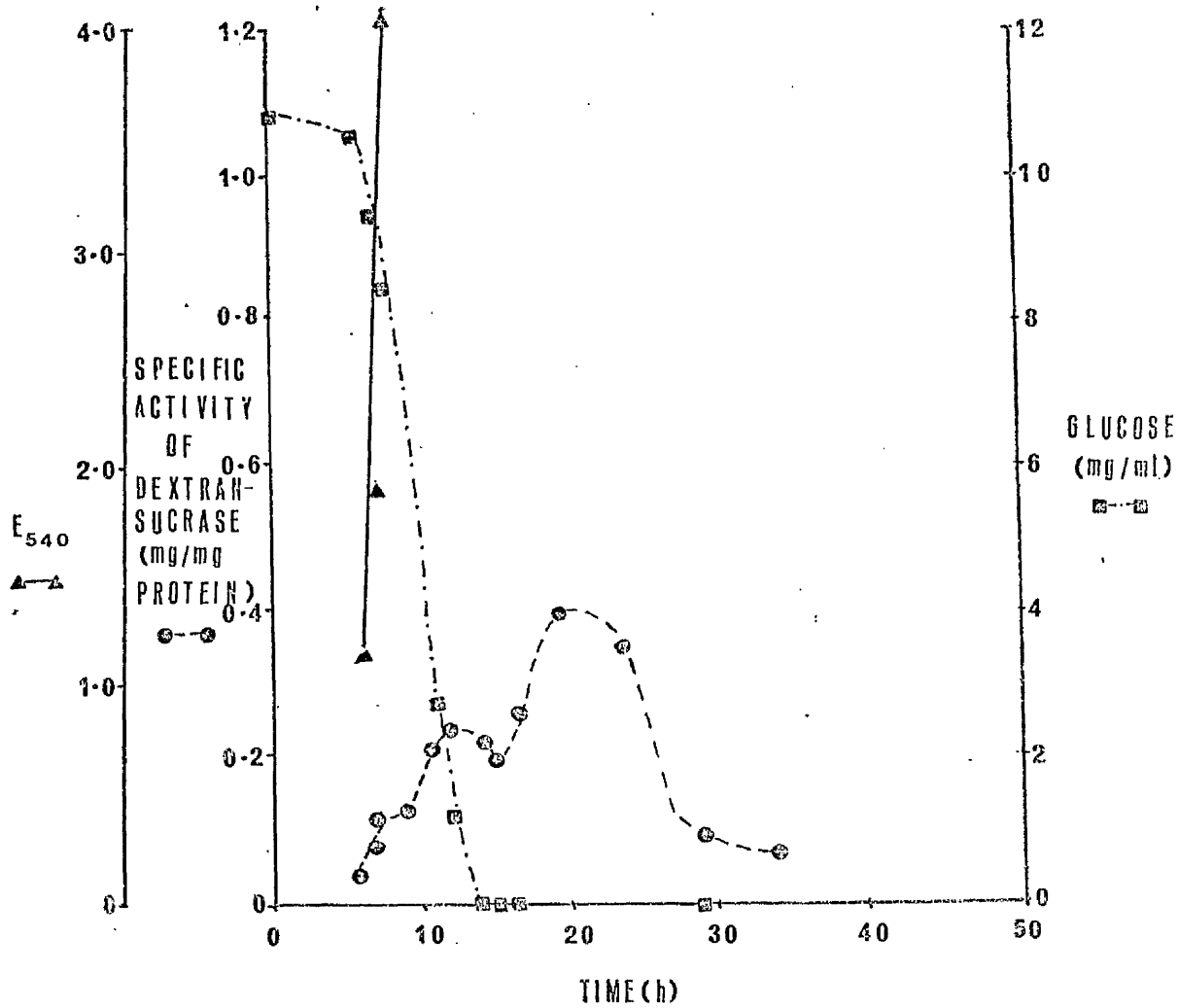


FIG. 3,11 DEXTRANSUCRASE PRODUCTION BY S. sanguis AT pH 6·6 ± 0·1

The experimental procedure was the same as that shown in Fig. 3.10 except that the pH was maintained at 6·6 ± 0·1.

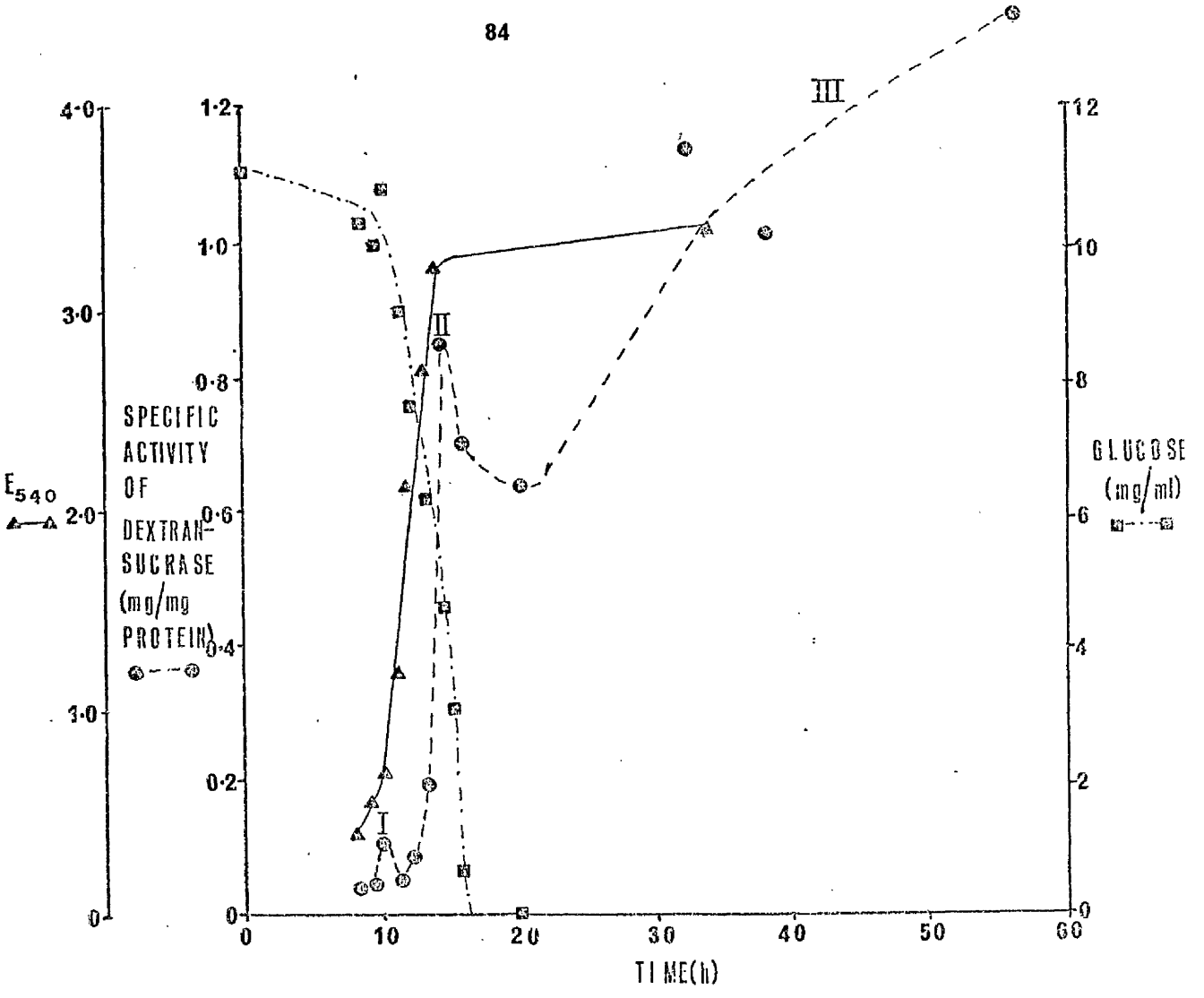


FIG. 3.12 DEXTRANSUCRASE PRODUCTION BY S. sanguis AT pH 7·1 ± 0·1.

The experimental procedure was the same as that shown in Fig.3.10 except that the pH was maintained at 7·1 ± 0·1. I, II and III indicate the three peaks of enzyme activity from which supernatant was harvested for the preparation of dextrans I, II and III - see Section 2.9.3.

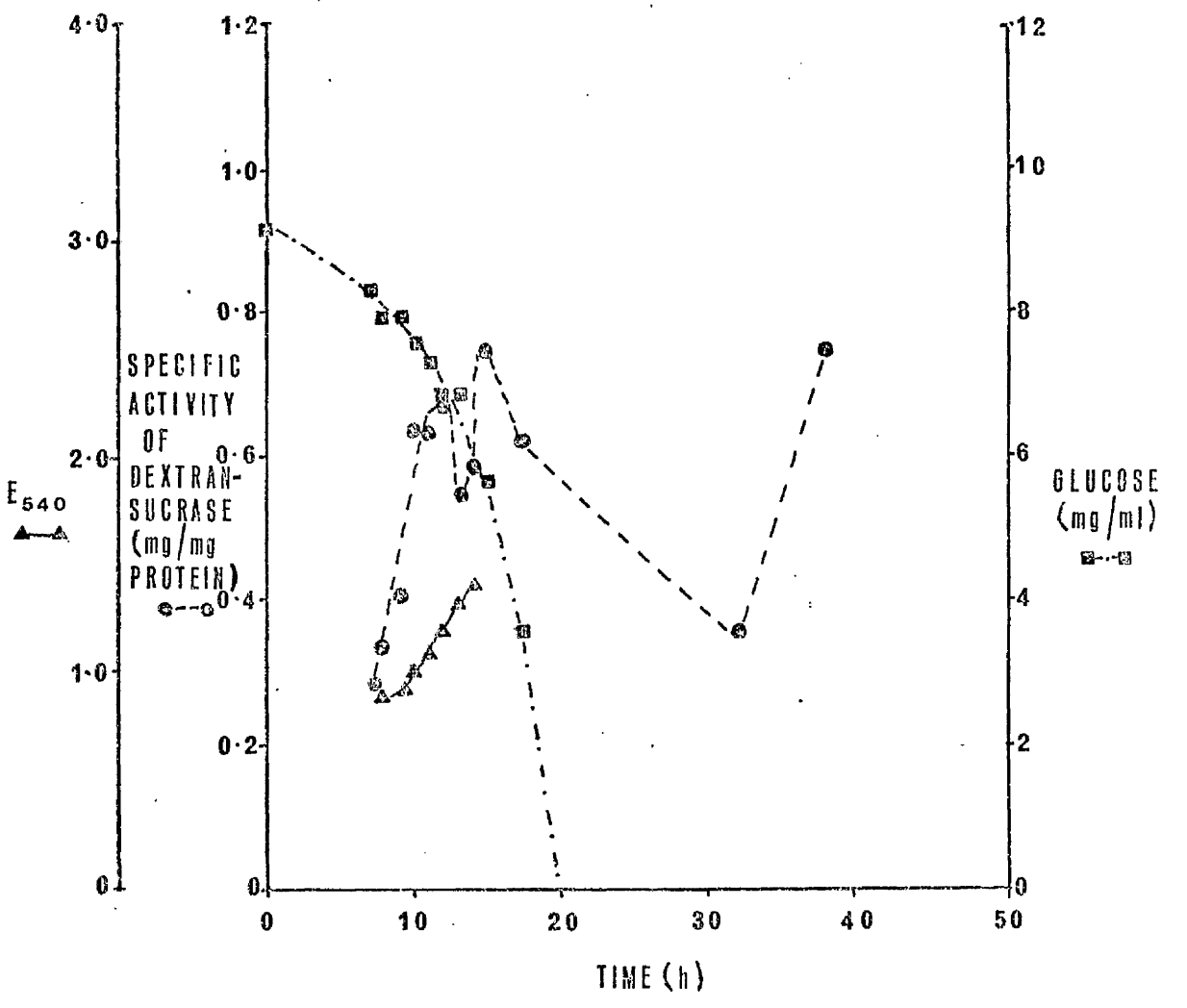


FIG.3.13 DEXTRANSUCRASE PRODUCTION BY S.sanguis AT pH 7·6 ± 0·1.

The experimental procedure was the same as that shown in Fig.3.10 except that the pH was maintained at 7·6 ± 0·1.

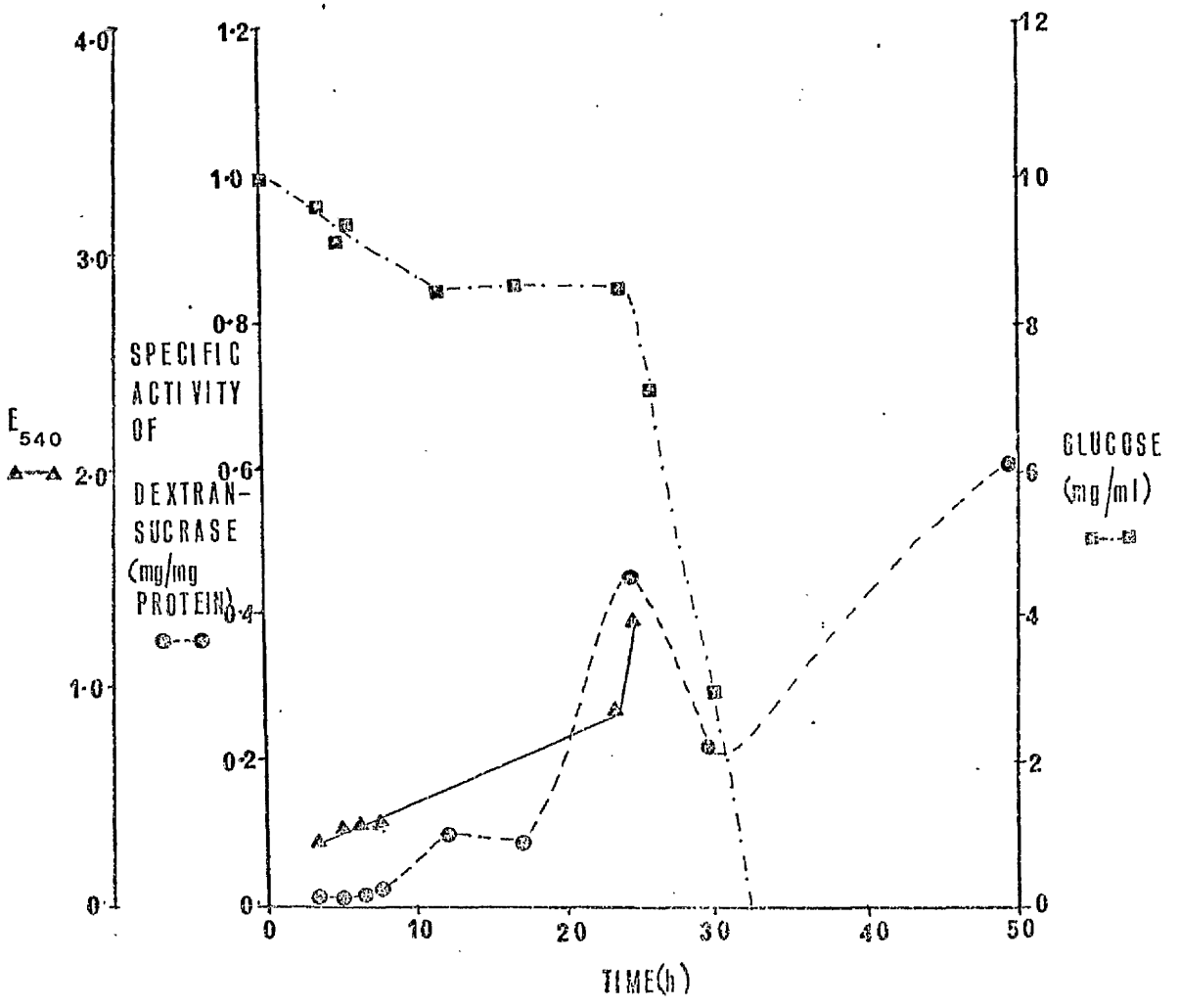


FIG.3.14 DEXTRANSUCRASE PRODUCTION BY S.sanguis AT pH 8·1 ± 0·1.

The experimental procedure was the same as that shown in Fig.3.10 except that the pH was maintained at 8·1 ± 0·1.

At all pH values studied, three distinct reproducible phases of dextransucrase production were apparent (Figs.3.10-3.14). The first occurred as the culture started to grow, the second when the growth rate was at a maximum and the last when growth had ceased and all glucose had been utilised (Figs.3.10-3.14). The significance of this production pattern will be discussed later (see Section 3.3.1). Dextransucrase activity expressed as specific activity in those experiments carried out under conditions of controlled pH and as dextran produced per ml supernatant in the experiments without pH control, was greatly increased under conditions of controlled pH. Maximum dextransucrase production occurred at pH $7{\cdot}1 \pm 0{\cdot}1$. Growth of S.sanguis was greatest at pH $6{\cdot}6 \pm 0{\cdot}1$, but dextransucrase activity was lower. The enzyme activity was higher in cultures maintained at pH values above 7 than at pH values below this figure. This could indicate that the enzyme is more stable at pH values above 7 than below (Figs.3.15-3.16).

3.2.4 The effect of contaminants. Unfortunately, despite careful sterilisation of all components of the fermenter flask assembly and the reduction to a minimum of manipulations and additions of components to the flask assembly once sterilised, contamination problems still arose. However, data collected from such contaminated cultures suggest that dextransucrase production is greatly increased (Figs.3.17-3.18). Comparison of the growth rates was not possible as aggregation occurred in the contaminated cultures. Isolation of one such contaminant (Staphylococcus aureas) and cultivation of it in Carlsson's diffusate medium without pH control revealed that it did not produce any precipitable carbohydrate when the supernatant was incubated with sucrose.

In one contaminated culture the contaminant was not identified, but appeared to be a member of the Pseudomonas species due to the production of an oily, fluorescent green pigment.

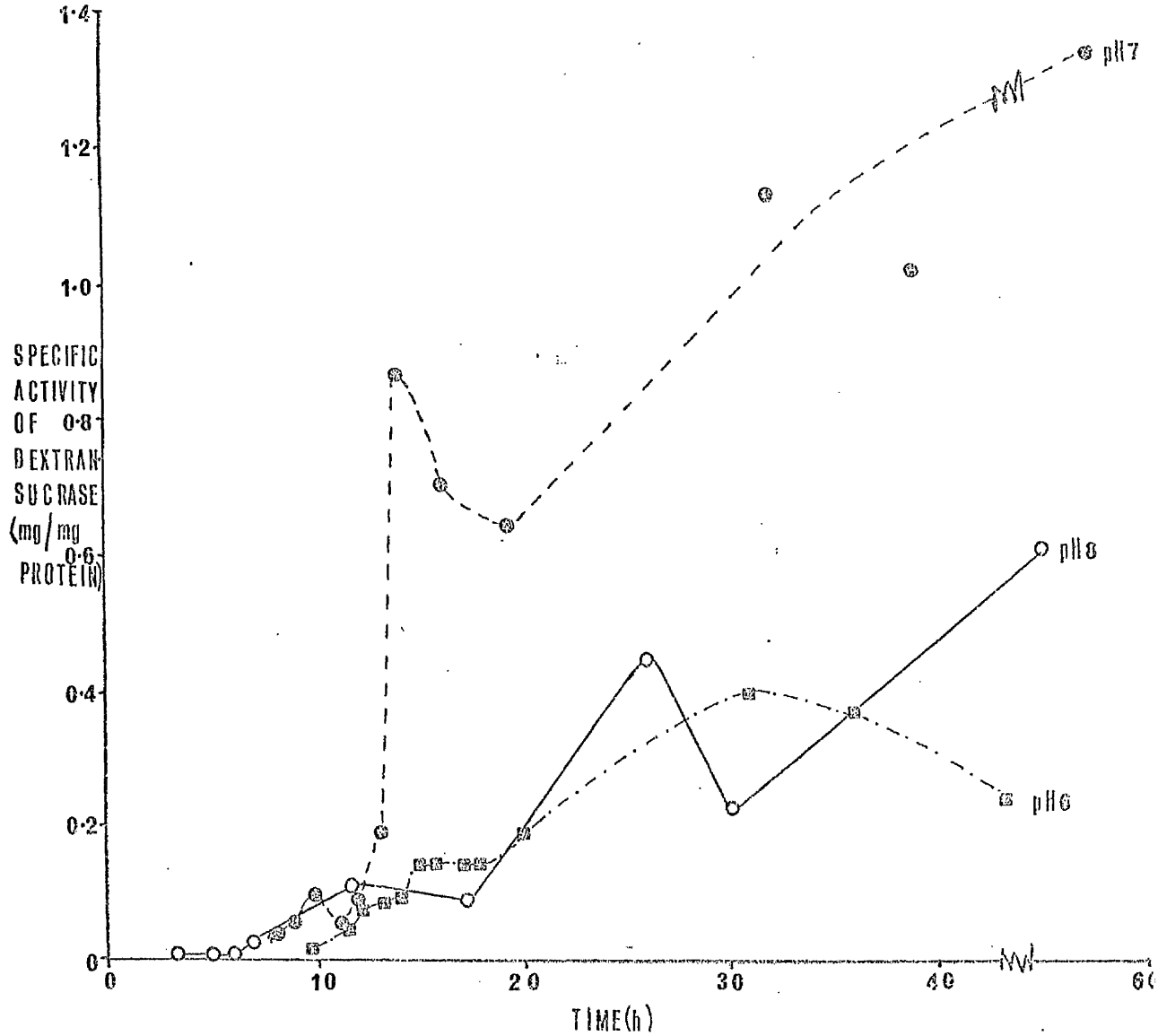


FIG.3.15 THE EFFECT OF pH CONTROL ON DEXTRANSUCRASE PRODUCTION AT pH 6, 7 AND 8.

The specific activities of dextransucrase produced when S.sanguis was grown at pH 6·1 ± 0·1, 7·1 ± 0·1 and 8·1 ± 0·1 are brought together in this figure (from Figs.3.10, 3.12 and 3.14) for purposes of comparison.

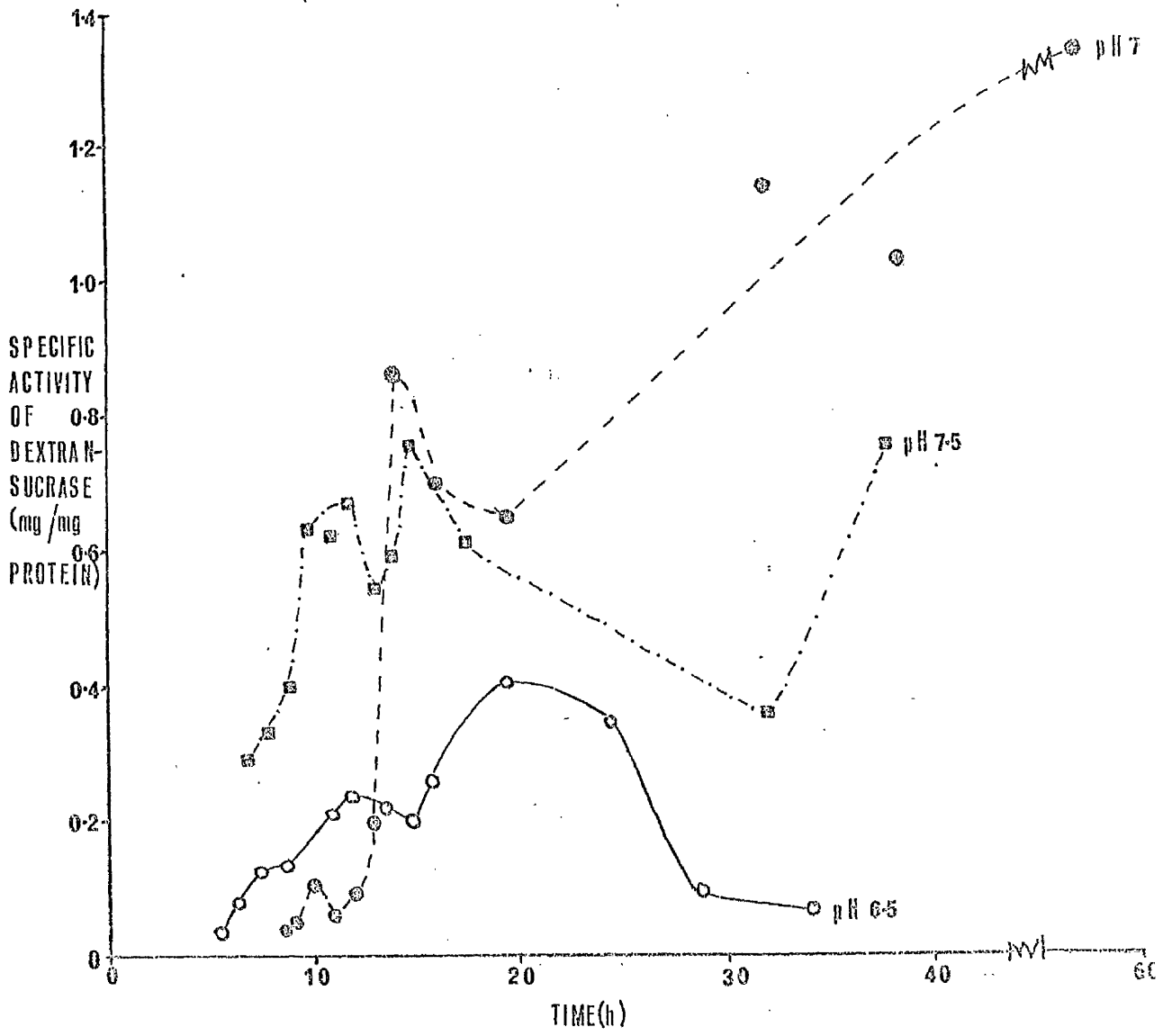


FIG.3.16 THE EFFECT OF pH CONTROL ON DEXTRANSUCRASE PRODUCTION AT pH 6·5, 7 AND 7·5.

The specific activities of dextransucrase produced when S.sanguis was grown at pH 6·6 ± 0·1, 7·1 ± 0·1 and 7·6 ± 0·1 are brought together in this graph (from Figs.3.11, 3.12 and 3.14) for purposes of comparison.

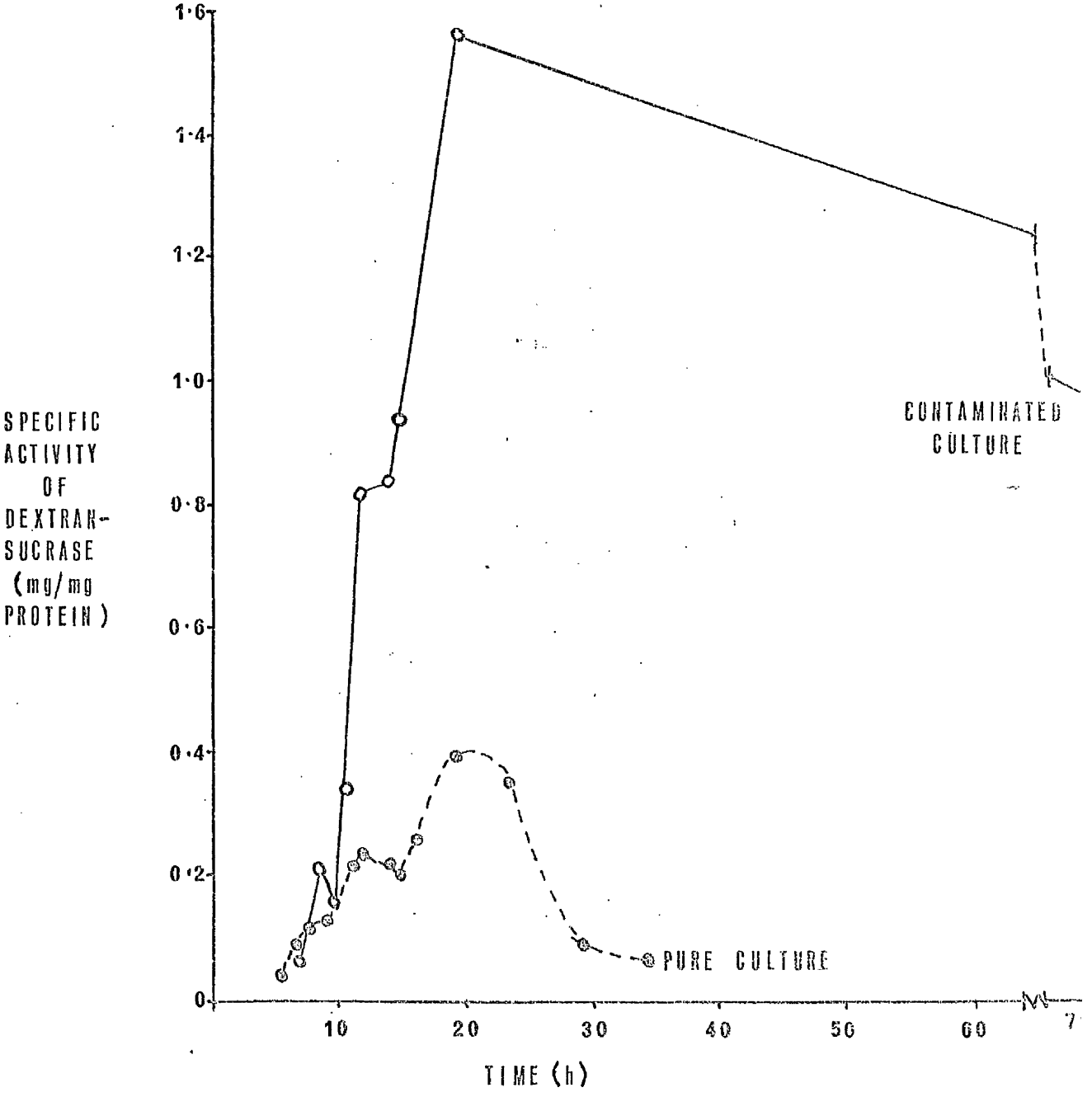


FIG.3.17 THE EFFECT OF CONTAMINATING BACTERIA ON THE PRODUCTION OF DEXTRANSUCRASE BY S.sanguis AT pH 6·6 ± 0·1.

200 ml Carlsson's diffusate medium was inoculated with a 2·5% 16h culture of S.sanguis at 37°C, with stirring. The pH was maintained at 6·6 ± 0·1. One culture was contaminated with Staphylococcus aureas at zero time. Aliquots of supernatant were assayed for dextransucrase activity as described in Section 2.15.2ii and for protein by the Lowry method. The specific activity of dextransucrase is the amount of precipitable carbohydrate, as measured by the phenol/H_2SO_4 method, per mg of protein.

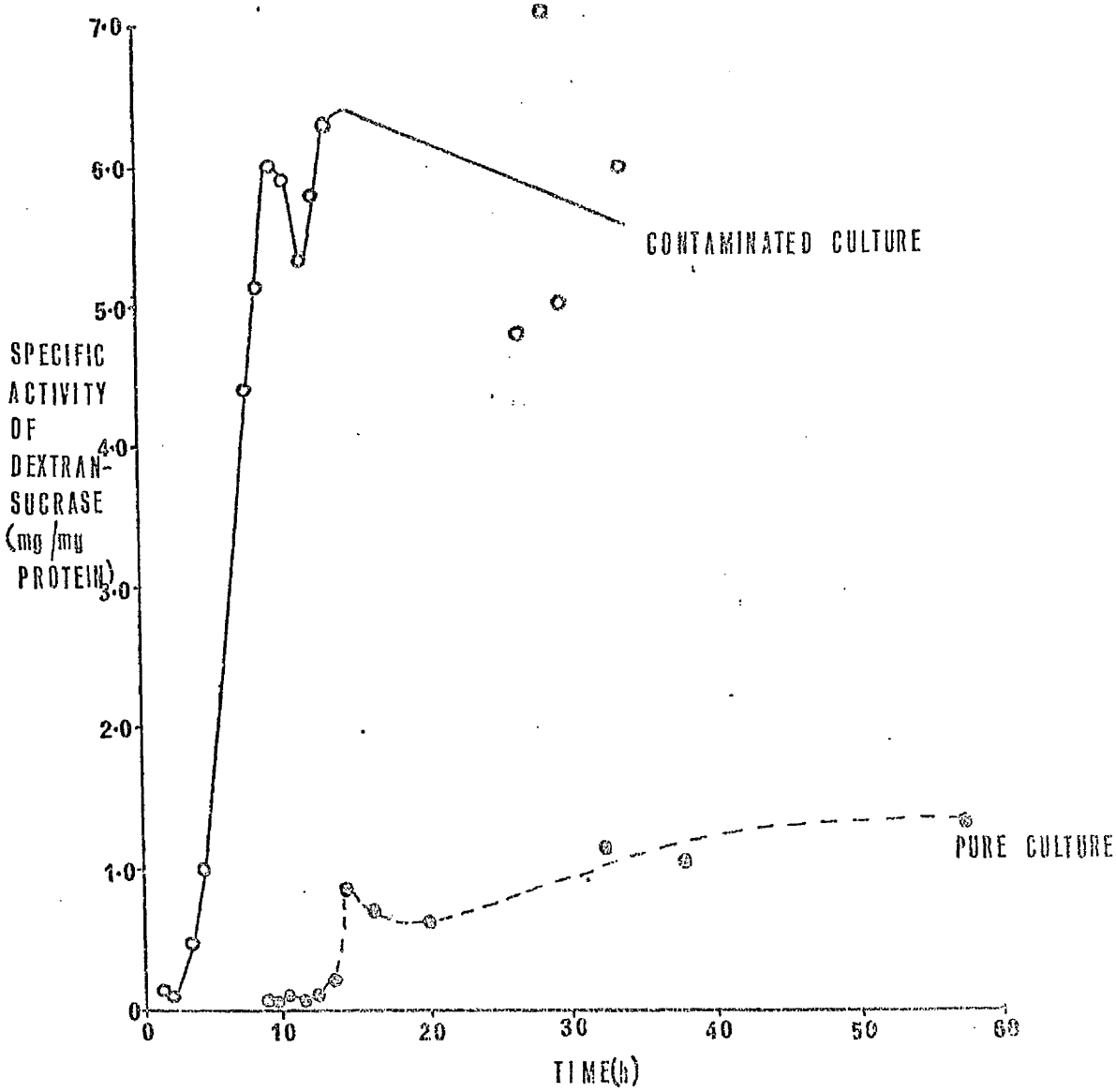


FIG.3.18 THE EFFECT OF CONTAMINATING BACTERIA ON THE PRODUCTION OF DEXTRANSUCRASE BY S.sanguis AT pH 7·1 ± 0·1.

The experimental procedure was the same as that shown in Fig.3.17 except that the pH was maintained at 7·1 ± 0·1. The contaminant was Staph.aurees.

The increase in dextransucrase activity in contaminated cultures (approximately 4 - 7 times that in uncontaminated cultures) became apparent when the degree of contamination was so low that it was not detectable by an examination of Gram stained slide preparations or plating out on blood agar e.g. In the experiment shown in Fig.3.17 contamination was not detected until the 14h sample by which time the enzyme activity had reached four times the value of the uncontaminated sample.

Re-examination of the inoculation culture at this stage revealed the presence of contaminants (previously undetected) indicating that contamination had been present at this stage.

3.2.5 Conclusions. Of those examined, a 2·5% 16h inoculum best fulfilled the requirement for a conveniently prepared inoculum which initiates rapid production of large amounts of dextransucrase (Fig.3.19).

The optimum pH for production of dextransucrase under the conditions employed was found to be in the region of $7{\cdot}1 \pm 0{\cdot}1$.

Growth of S.sanguis in mixed culture with Staph. aureas increased the yield of dextransucrase. It is suggested that this might be due to the production or removal of some factor by Staph. aureas which promotes dextransucrase production by S.sanguis.

The great increase in dextransucrase activity observed at very low levels of contamination suggests that any such factor produced by Staph. aureas must be active in very low concentration. This factor might be catalase, which has recently been shown to promote growth of S.sanguis (see Section 1.5.2). when grown in the presence of oxygen.

Staph. aureas and all members of the Pseudomonas species produce catalase (Topley and Wilson, 1966).

However, further work is necessary to investigate this phenomenon.

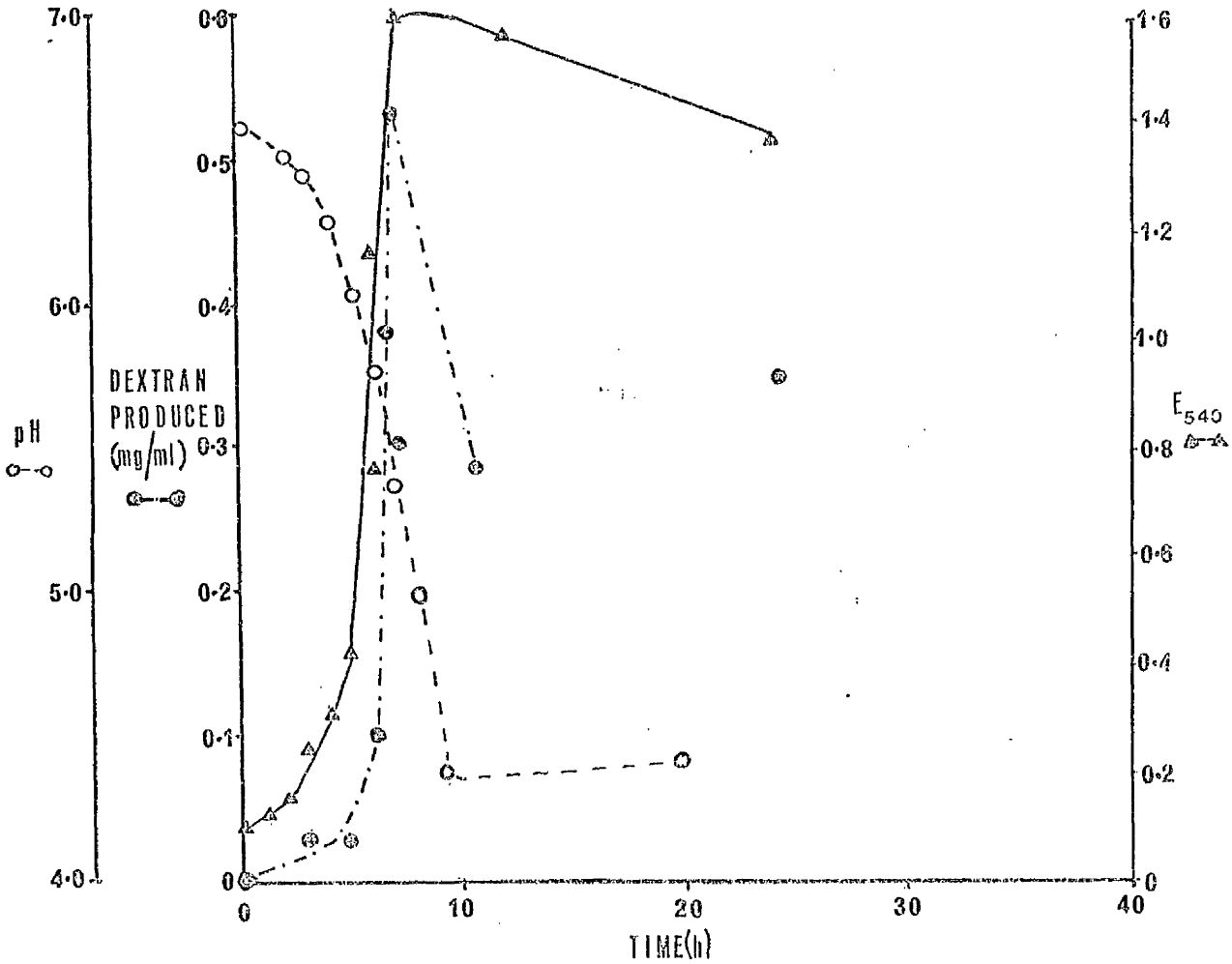


FIG.3.19 THE EFFECT OF A 2·5% INOCULUM OF A 16h CULTURE OF S.sanguis ON SUBSEQUENT GROWTH AND DEXTRANSUCRASE PRODUCTION.

200 ml Carlsson's diffusate medium was inoculated with 5 ml of a 16h culture of S.sanguis and maintained at 37°C, with shaking. Samples were removed at known time intervals and the pH (o - - o) and extinction at 540 nm (Δ——Δ) measured. Aliquots of supernatant were assayed for dextransucrase (o —·— o) as described in Section 2.15.2ii.

3.3 DEXTRANSUCRASE PRODUCTION PATTERNS

At all pH values studied, the change in specific activity of dextransucrase with time was seen to follow a triphasic pattern (Figs.3.15-3.16). The results of these experiments are recorded on two separate graphs for the sake of simplicity.

3.3.1 Conclusions. The elaboration of three peaks of dextransucrase production could be due to:-

(i) the presence of proteases which break down the dextransucrase, perhaps at varying rates, as it is formed or dextranases which hydrolyse the dextran formed by the dextransucrase in the assay procedure.

(ii) fluctuations in the level of unknown additional factor/s necessary for dextran production, or changes in the concentration of an activator or inhibitor.

(iii) factors influencing release of the enzyme from the cells, e.g. intracellular and cell membrane bound enzyme may be released by lysis of the cells upon death (See Section 1.4.4.).

(iv) the elaboration of a multi-enzyme system.

Proteases and dextranases would be expected to cause a reduction in the amount of dextran formed, but not to alter the structure of the dextran. Further evidence has been put forward by other workers which indicates that S.sanguis 804 does not produce dextranase (See Section 1 4.4.). Similarly factors necessary for dextran production or release of dextransucrase from the cell would be expected to affect the amount of dextran only.

However, if the elaboration of a multi-enzyme system were responsible for the peaks of enzyme activity, changes in the structure of the dextran would be expected with each enzyme or combination of enzymes present in the culture fluid.

In order to investigate whether the structure of the dextran changes from one enzyme peak to the next or not, it would be necessary to produce dextran from each of the three phases of enzyme production, prepare dextran from the culture supernatant and, after characterising the precipitate as dextran, to examine it structurally. Dextrans I, II and III were prepared from peaks I, II and III respectively of a culture grown at pH 7·1 ± 0·1 (Fig.3.12). It would also be of interest, to attempt purification of enzyme prepared from each phase in order to see if more than one enzyme could be distinguished.

3.4 CHARACTERISATION OF THE PRODUCTS OF THE ACTION OF DEXTRANSUCRASE ON SUCROSE

3.4.1 Gas-liquid chromatography. The chromatographs for the preparations from all three phases of enzyme activity revealed two major peaks which corresponded with those obtained for a glucose standard (Fig.3.20). Dextrans I and II produced a minor peak corresponding to a trace of fructose and Dextran I a minor unidentified peak. The chromatographs indicate that all three preparations contain glucose as the principal carbohydrate and that they contain at least 90% glucose.

3.4.2 Analysis of acidic hydrolysates. The three preparations consist of 88·6%, 92·8% and 82·9% carbohydrate respectively when measured by the phenol/H_2SO_4 method and 90·5%, 97·6% and 83·1% glucose when measured by the glucose oxidase method (Table 3.1). However, of the carbohydrate fraction of the preparations, all three consist entirely of glucose.

Glucans of known structure were included for purposes of comparison (Table 3.1) and glucose and dextran T2000 standards were also assayed. The glucans are all homopolymers of glucose which had been dried to constant weight to preclude any error due to moisture content. Nigeran appeared to be contaminated with some carbohydrate other than glucose (Table 3.1). Laminarin, amylopectin, glycogen, mutan 1, mutan 2 and dextran T2000 consisted of 90-100% carbohydrate, when measured by the phenol/H_2SO_4 method and this carbohydrate was glucose. S-glucan prepared from A.nidulans by the method of Zonneveld (1971) was contaminated with a large amount of non-carbohydrate material.

FIG. 3.20 G.L.C. OF PRODUCTS OF ACTION OF DEXTRANSUCRASE ON SUCROSE.

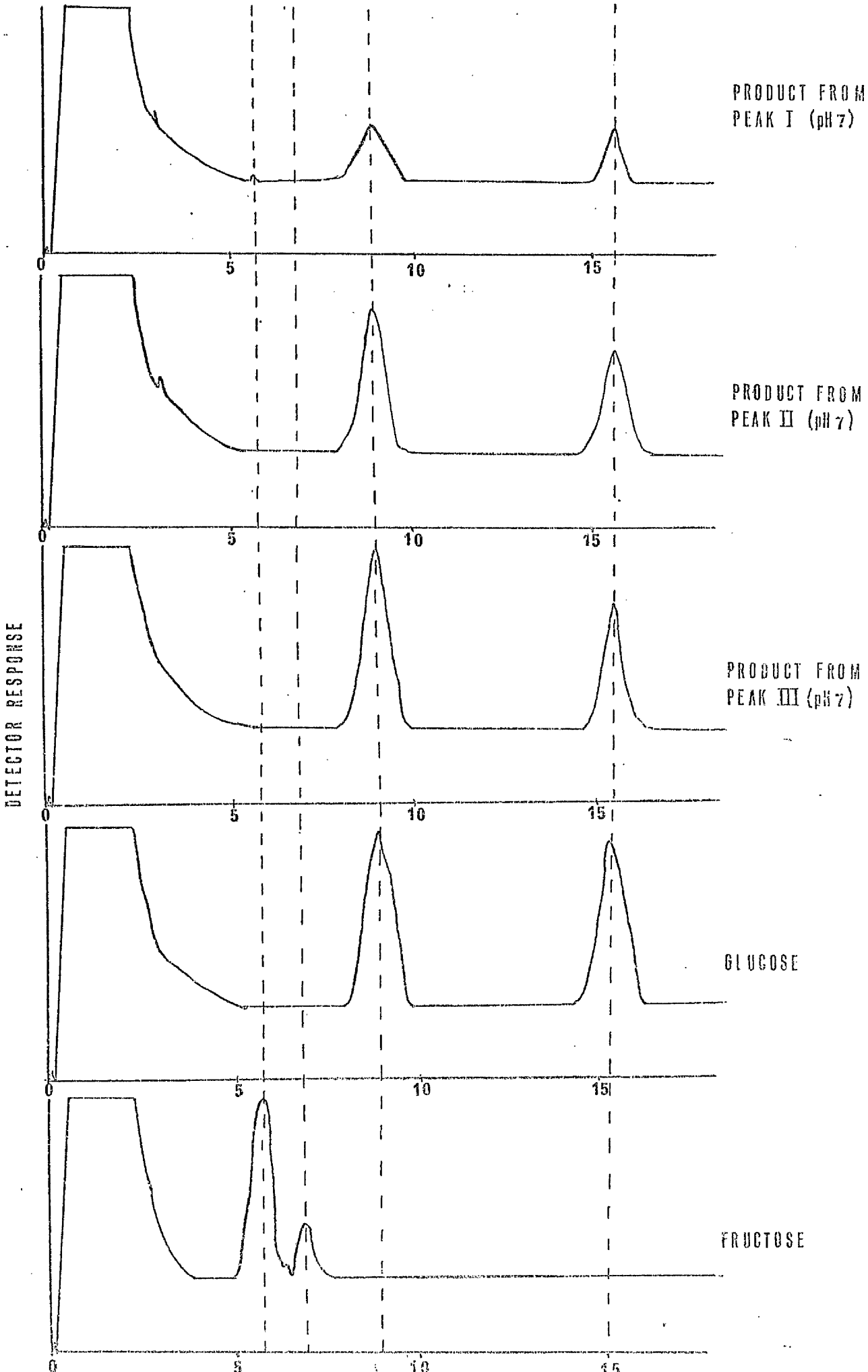

PRODUCT FROM PEAK I (pH 7)
PRODUCT FROM PEAK II (pH 7)
PRODUCT FROM PEAK III (pH 7)
GLUCOSE
FRUCTOSE
DETECTOR RESPONSE
0
5
10
15

TABLE 3.1. ACIDIC HYDROLYSIS OF GLUCANS

	% (w/w) carbohydrate	% (w/w) glucose	% (w/w) carbohydrate as glucose
Glucose	100·0	100·0	100·0
Dextran I	88·6	90·5	101·6
Dextran II	92·8	97·6	104·6
Dextran III	82·9	83·1	100·2
Laminarin	90·7	92·8	101·9
Nigeran	96·0	85·1	88·2
Amylopectin	95·5	95·0	100·0
Glycogen	98·6	100·0	100·9
Dextran T2000	110·7	98·1	88·2
Mutan 1 (Schweizersche)	95·2	90·6	95·2
Mutan 2 (GMZ 176)	104·8	101·1	96·1
S-glucan	26·3	24·6	92·5

However, the carbohydrate in this preparation was almost entirely glucose.

3.4.3 Specific aggregation of S.mutans. The addition of dextran to washed cells of S.mutans causes specific aggregation of the cells (see Section 1.2.5.). This procedure can be used to detect the presence of dextran.

Dextran I, II, III and T2000 and mutan 1 all initiated aggregation of the cells immediately after their addition. Amylopectin, glycogen and glucose did not aggregate the cells even after a 24h. incubation period. No auto-agglutination was observed in the controls. Aggregation was not immediately apparent in the preparation containing mutan 2 but was observed after 24h. incubation. Mutan 2, which is a flaky, white solid, is only slightly soluble in the buffer and the amount of mutan 2 available to the cells may be too small to induce aggregation. The other dextran preparations were finely divided white powders offering a larger surface area to the cells. Aggregation might, therefore, be expected to occur more rapidly for these preparations than for mutan 2.

(i) Electron microscopy. The electron micrographs of the aggregated cells revealed clumps of coccoid organisms coated with an electron-dense layer. This layer appeared to consist of globules approximately 0.025μ in diameter and short 'bridges' consisting of chains of these globules appeared to be linking cells in some of the preparations (Plates 3,1-3,4). The control cells which were not aggregated did not possess such a coating.

3.4.4 Conclusions. The gas-liquid chromatographs and acidic hydrolysate analyses revealed that the products from peaks I, II and III were at least 80% carbohydrate and that virtually all of the carbohydrate was glucose. The close agreement between glucose and total carbohydrate assays suggests that the contaminating material is not carbohydrate and will not interfere with Cybulska and Pakula's modified dextransucrase assay. The contaminating material may be protein which is precipitated under conditions similar to

PLATES ELECTRON MICROGRAPHS OF AGGREGATED CELLS OF S.MUTANS.

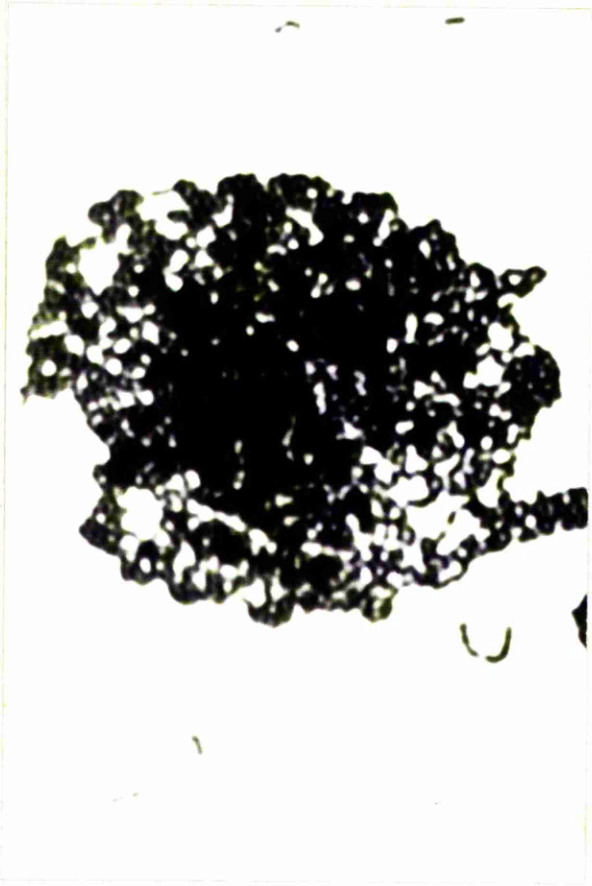
Washed cells of S.mutans OMZ 176 (10^9 cells/ml. 0·067M sodium phosphate buffer, pH 8·0) aggregated in the presence of dextran T2000 or dextran III. The aggregated cells were washed in buffer and distilled water, and dried in air on electron microscope grids. They were then examined under the electron microscope.

Plate 3.1. Aggregated cells of S.mutans stained by Gram's method as seen under the light microscope. Magnification x 1,000.

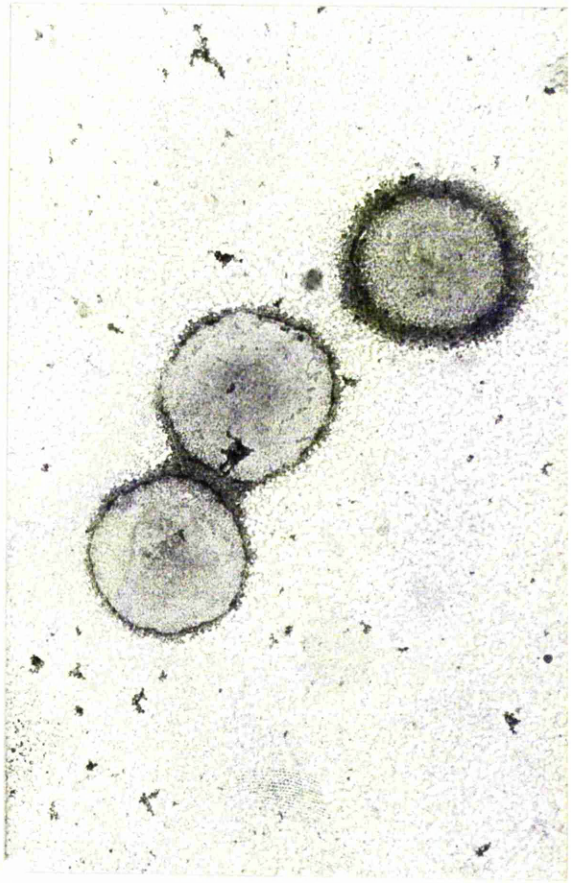
Plate 3.2 Cells of S.mutans singly and in pairs coated by a fibrillar electron dense material. Magnification x 20,000.

Plate 3.3 Dextran T2000 aggregated cells of S.mutans coated by a globular electron dense material. Chains of globules are also visible. Magnification x 45,000.

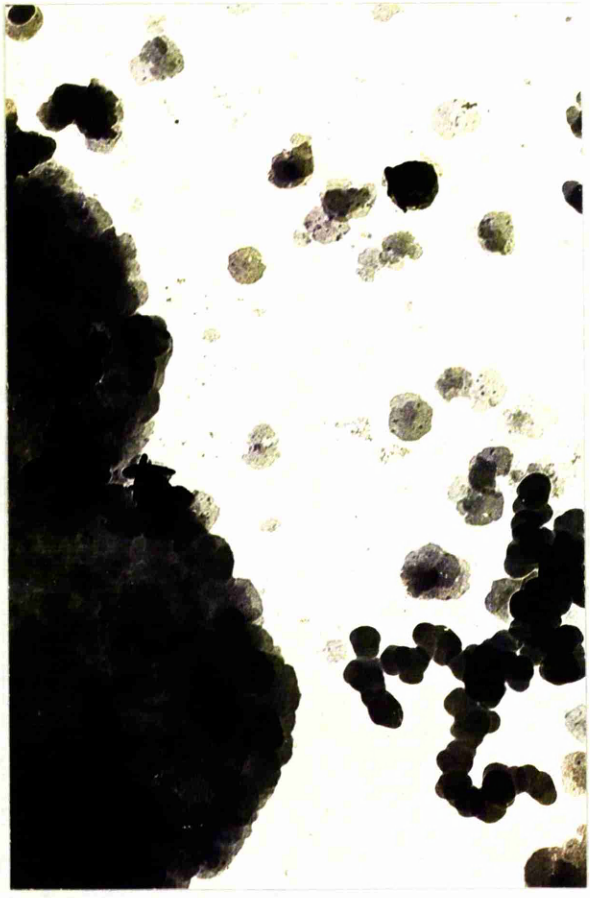
Plate 3.4 Dextran III aggregated cells of S.mutans demonstrating the presence of 'bridges' of electron dense material linking cells. Magnification x 40,000.



3.1 10μm



3.2 0·5μm



3.3 0·2μm

3.4 0·2μm

those required for precipitation of dextran.

The aggregation of cells of S.mutans in the presence of each of the three preparations indicates that all three preparations are "dextran-like".

3.5. STRUCTURAL EXAMINATION OF DEXTRANS

3.5.1 Infra-red spectroscopy. Fig.3.21 demonstrates the appearance of the infra-red spectrum obtained for dextran T2000. The peaks indicative of α- and β-glucans occur in the region 600 - 1000 cm^{-1} and this region from the spectrum for each glucan has been reproduced in Figs.3.22-323.The glucans were all homopolymers of glucose with known structures which had undergone acidic hydrolysis (Section 2.10.2) to determine their degree of purity. Glucans with different proportions of α-(1→6)-, α-(1→3)-, α-(1→4), β-(1→3) and β-(1→6)- linkages were included for comparison with the preparations from peaks I, II and III in order to gain some information about the types of linkage present in these preparations.

Comparison of the spectra reveals variations between them in the presence or absence of the peaks and in the sizes of those peaks. Absorbance in the region 760-775 and 905-930 is attributed to the presence of α-gluco-pyranose ring breathing and can be detected in all the glucans except laminarin (a β-glucan containing β-(1→3)- and β-(1→6)- linkages in the ratio 1 : 1). This glucan has a peak in the region of 780 cm^{-1} which is indicative of β-glucopyranose ring breathing and at 818 cm^{-1} which typifies a β-linkage. The peak obtained in the region 837-850 for all of the glucans except laminarin is due to the presence of an α-linkage. It is now generally accepted that the presence of α-(1→3)- linkages as branch points is verified by absorbance at 790 cm^{-1} (Heyn, 1974) and such linkages are discernable for nigeran (contains α-(1→3)- and α-(1→4)- linkages in the ratio 1 : 1), dextran I, dextran II and mutan 1. The presence of such a peak for dextran III is not completely clear but, if it is present, it is certainly less

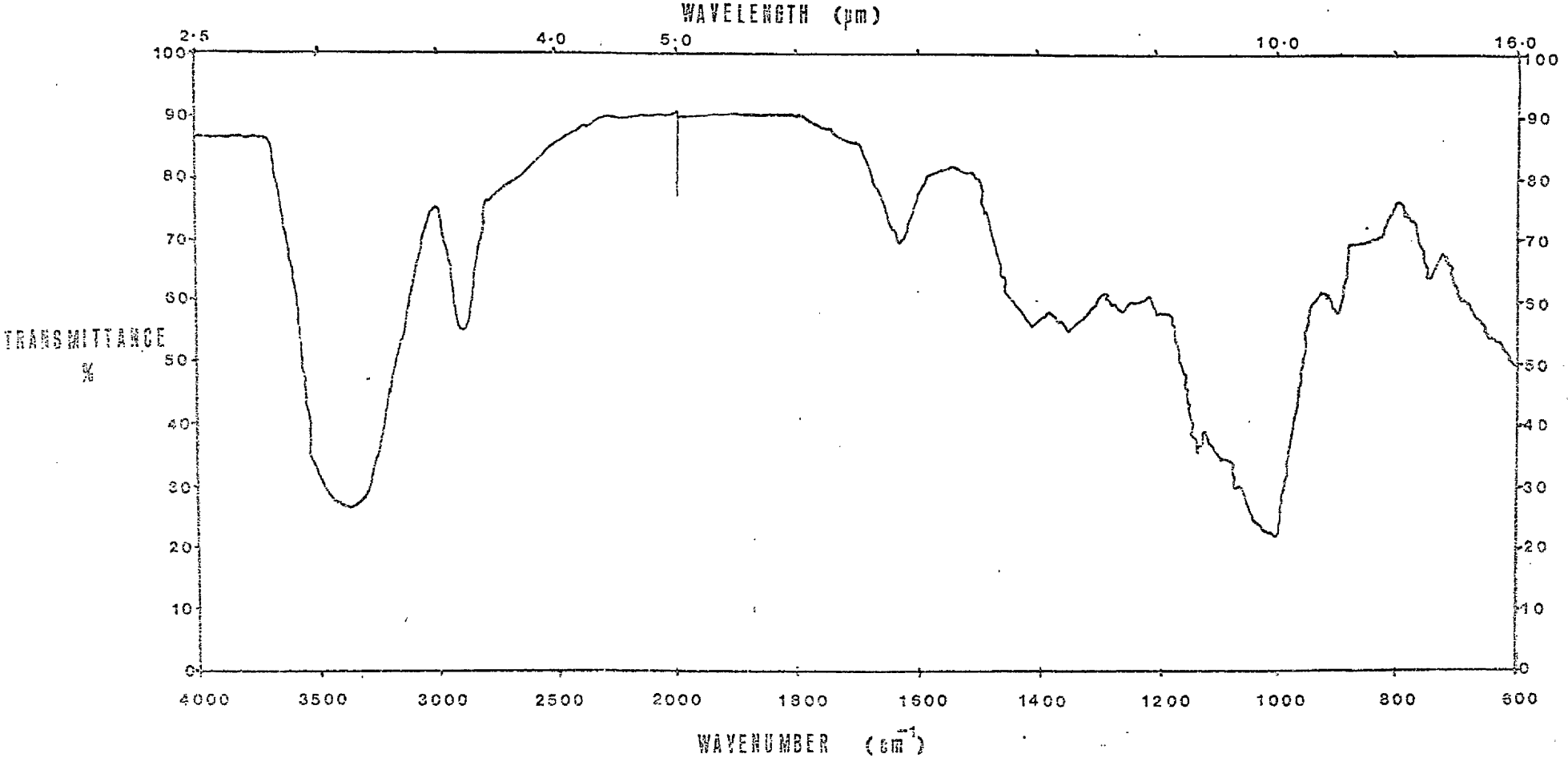


FIG.3.21 INFRA-RED SPECTRUM OBTAINED FOR DEXTRAN T2000

The dextran was pressed into a KBr disc (0·9 mg/100 mg) and scanned at medium speed in a Pye 225 spectrophotometer.

FIG.3.22 INFRA-RED SPECTRA OBTAINED FOR GLUCANS IN THE REGION 600 - 1000 CM^{-1} (WAVENUMBER.)

The experimental procedure was the same as in Fig.3.21. That section of each spectrum between wavenumbers 1000 and 600 and above the line indicating 50% transmittance is illustrated. The rest of the spectrum has been omitted for simplification.

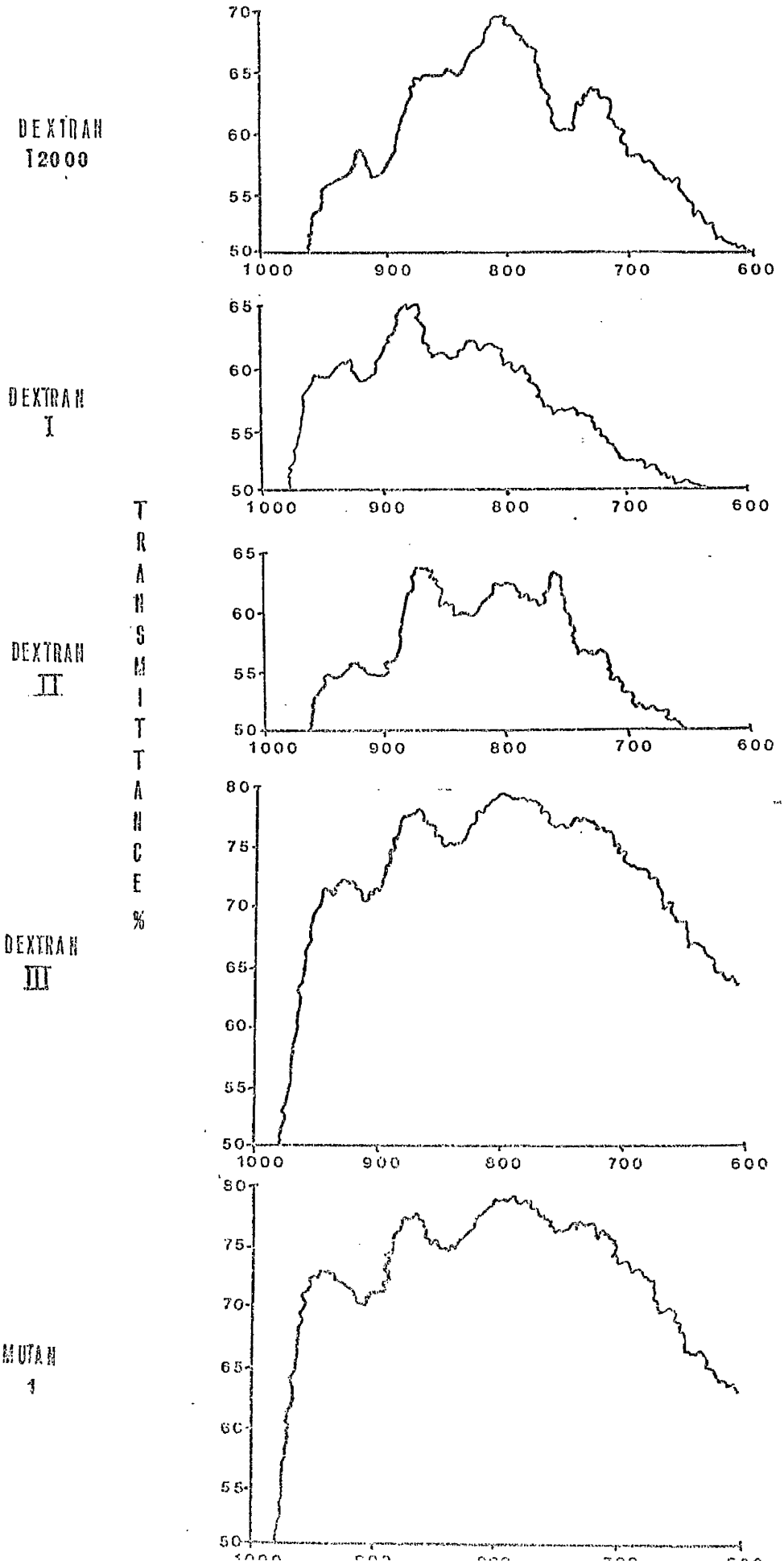
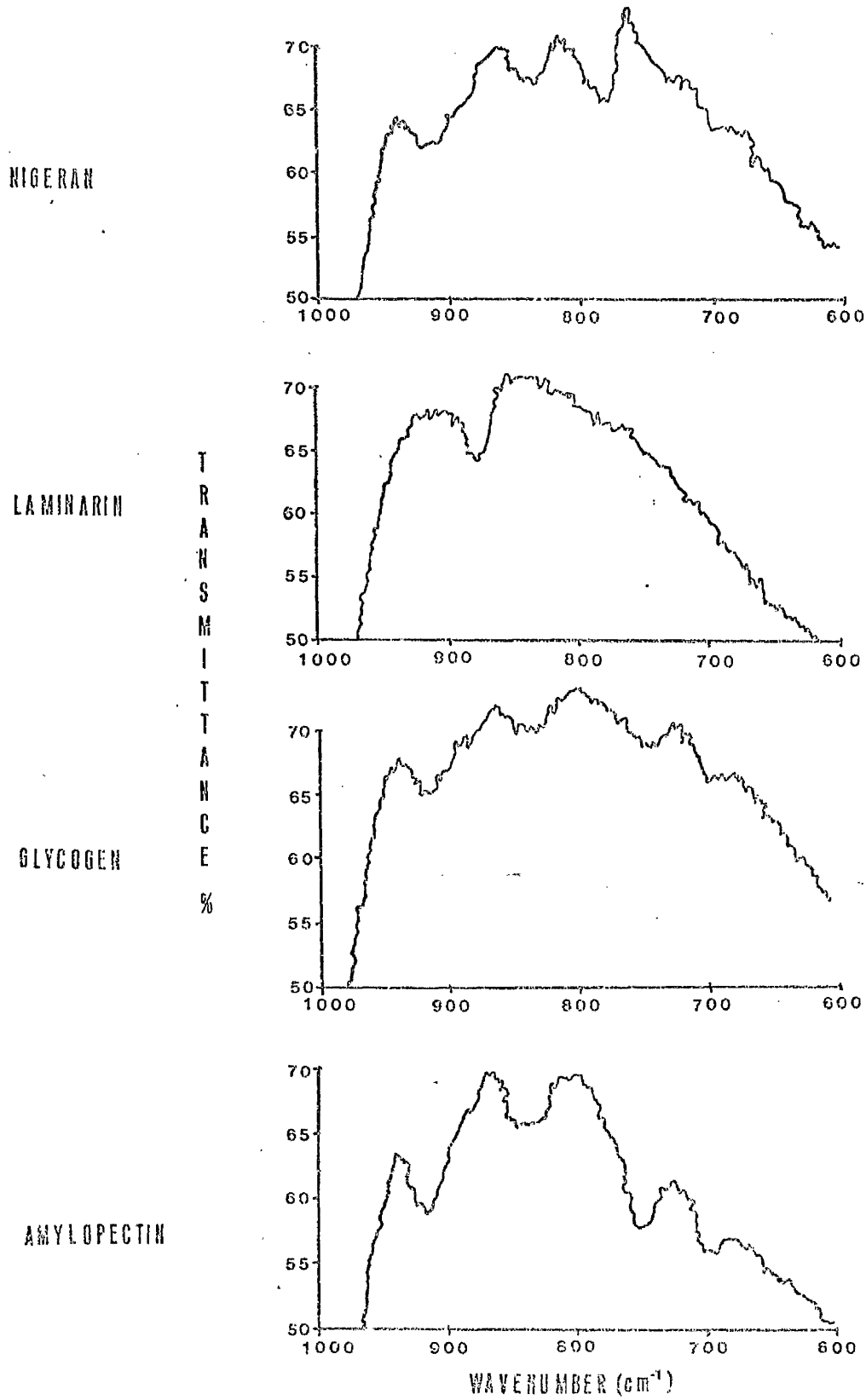

DEXTRAN T2000
DEXTRAN I
DEXTRAN II
DEXTRAN III
MUTAN 1
TRANSMITTANCE %
70
65
60
55
50
1000
900
800
700
600
65
60
55
50
1000
900
800
700
600
65
60
55
50
1000
900
800
700
600
80
75
70
65
60
55
50
1000
900
800
700
600
80
75
70
65
60
55
50

FIG. 3.23 INFRA-RED SPECTRA OF GLUCANS.

The experimental procedure was the same as in Fig.3.21. A section of each spectrum only is illustrated, as in Fig.3.22.


NIGERAN
LAMINARIN
GLYCOGEN
AMYLOPECTIN
TRANSMITTANCE %
70
65
60
55
50
1000
900
800
700
600
WAVENUMBER (cm⁻¹)

marked than that for dextrans I and II. The spectra for dextran III and mutan (84% α-(1→3)- linkages, 16% α-(1→6)- linkages) - Guggenheim, 1969, are very similar.

No information is at present available regarding the position of peaks indicative of α-(1→3)- linkages in the backbone of the dextran chain.

3.5.2 Enzymic degradation. Figs.3.24-3.25 show the increase in reducing power which was obtained on enzymic degradation of glucans with mutanase and dextranase respectively. Mutanase rapidly hydrolysed laminarin, amylopectin and glycogen suggesting contamination of the enzyme preparation by α-amylase and enzymes capable of degrading laminarin. Mutans 1 and 2 were broken down to a lesser extent (Fig.3.24) but, of the three dextran preparations only Dextran I was degraded and this was only to a very limited extent. Dextran T2000 was not hydrolysed. Dextranase hydrolysed, dextran T2000, dextrans I, II and III and the two mutan preparations. Of the three dextrans prepared from S.sanguis supernatant, dextran II was most readily hydrolysed followed by dextran I and then dextran III which behaved in a very similar manner to mutan 2 (Fig.3.25). α-amylase failed to hydrolyse any of the dextrans or mutan but readily degraded glycogen and amylopectin under the same conditions.

3.5.3 Periodate oxidation. Periodate consumption by dextran T2000, and mutans 1 and 2 reached completion within 24h. Dextrans I, II and III took slightly longer, reaching completion within 48h (Fig.3.26).

Initially, dextran II was more rapidly oxidised than dextran III but, by the time oxidation had reached completion, dextrans II and III had consumed equal quantities of periodate.

The calculation of the proportions of linkage types in dextran molecules from the consumption of periodate is explained in Section 1.3.4.i. Using this calculation, dextran T2000 was found to consist of 102% '1 - 6 like'

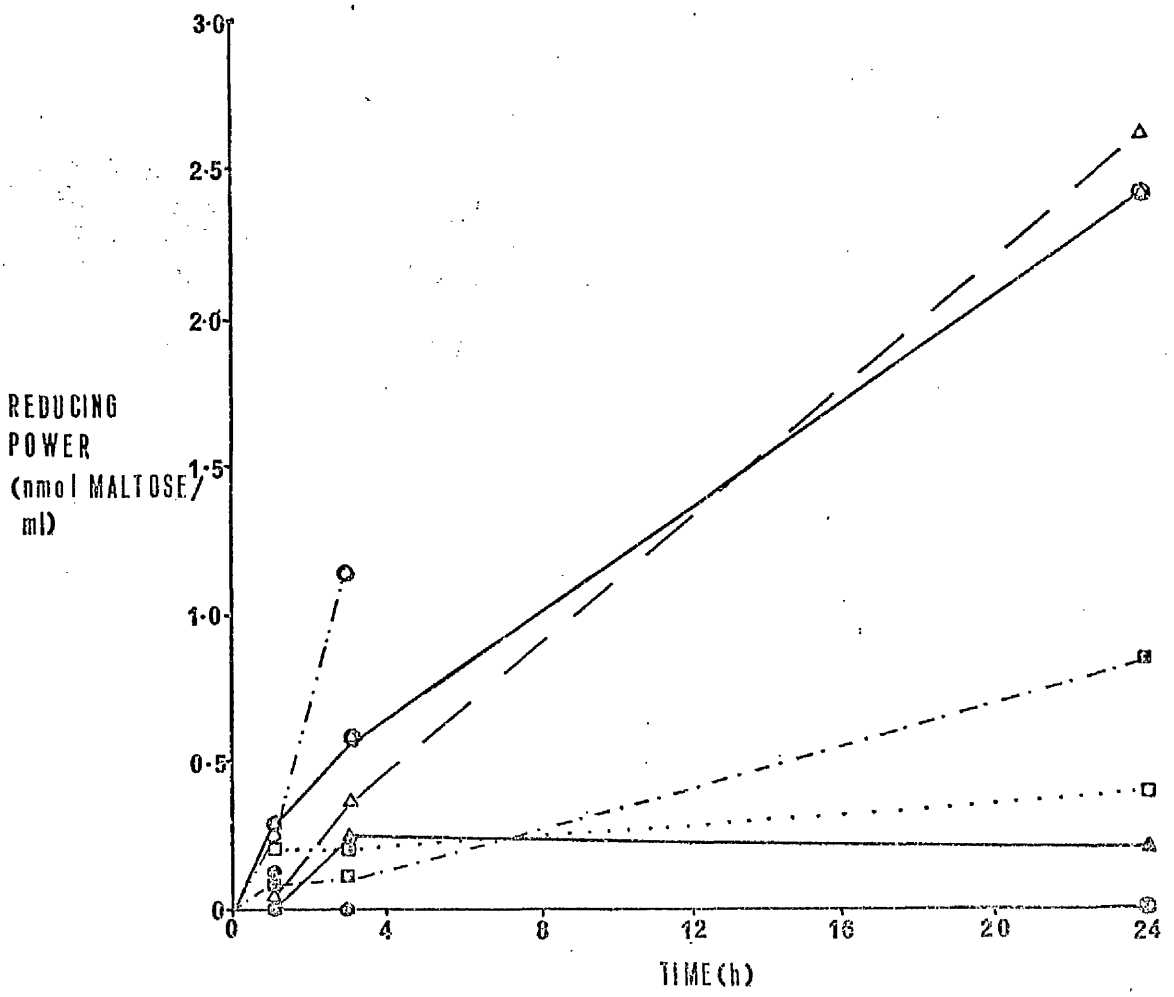


FIG.3.24 ENZYMIC DEGRADATION OF GLUCANS BY MUTANASE

The assay mixture contained 1 ml mutanase (50 µg/ml) in 0·2M sodium acetate buffer, pH 4·5 and 1 ml glucan solution (5 mg/ml) in the same buffer. Aliquots (0·2 ml) were removed at known time intervals and assayed for reducing sugar as maltose by the Somogyi method. Laminarin (o -·-·-o), amylopectin (Δ —— Δ), glycogen (◎——◎), mutan 1 - Schweizersche (□···□), mutan 2 - OMZ 176 (▣ -·-·- ▣), dextran I (Δ - Δ), dextran II (◎ - - ◎), dextran III (o - o) and dextran T2000 (Δ —·· —— Δ) were the glucans used. Dextran T2000 was not degraded.

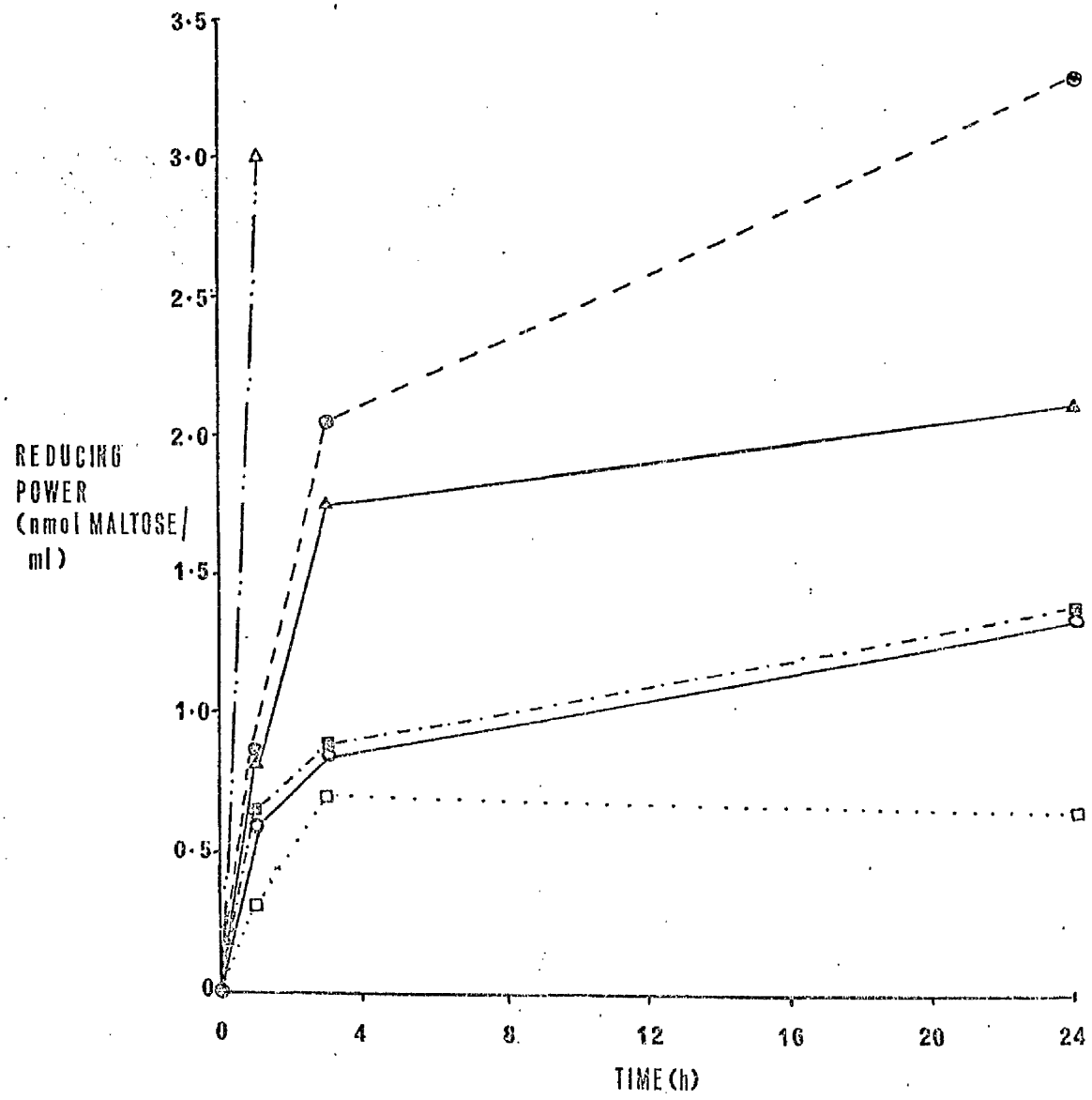


FIG.3.2 ENZYMIC DEGRADATION OF GLUCANS BY DEXTRANASE.

The assay mixture contained 1 ml dextranase (6·6µg/ml) in 0·2M sodium acetate buffer, pH 4·5 and 1 ml glucan solution (5 mg/ml) in the same buffer. Aliquots (0·2ml) were removed at known time intervals and assayed for reducing sugar, as maltose, by the Somogyi method. Mutan 1 (□···□), mutan 2 (▣·-·▣), dextran I (△——△), dextran II (⊙- - -⊙), dextran III (○——○) and dextran T2000 (△—— · · ——△) were the glucans degraded. Laminarin, nigeran, amylopectin and glycogen were not degraded.

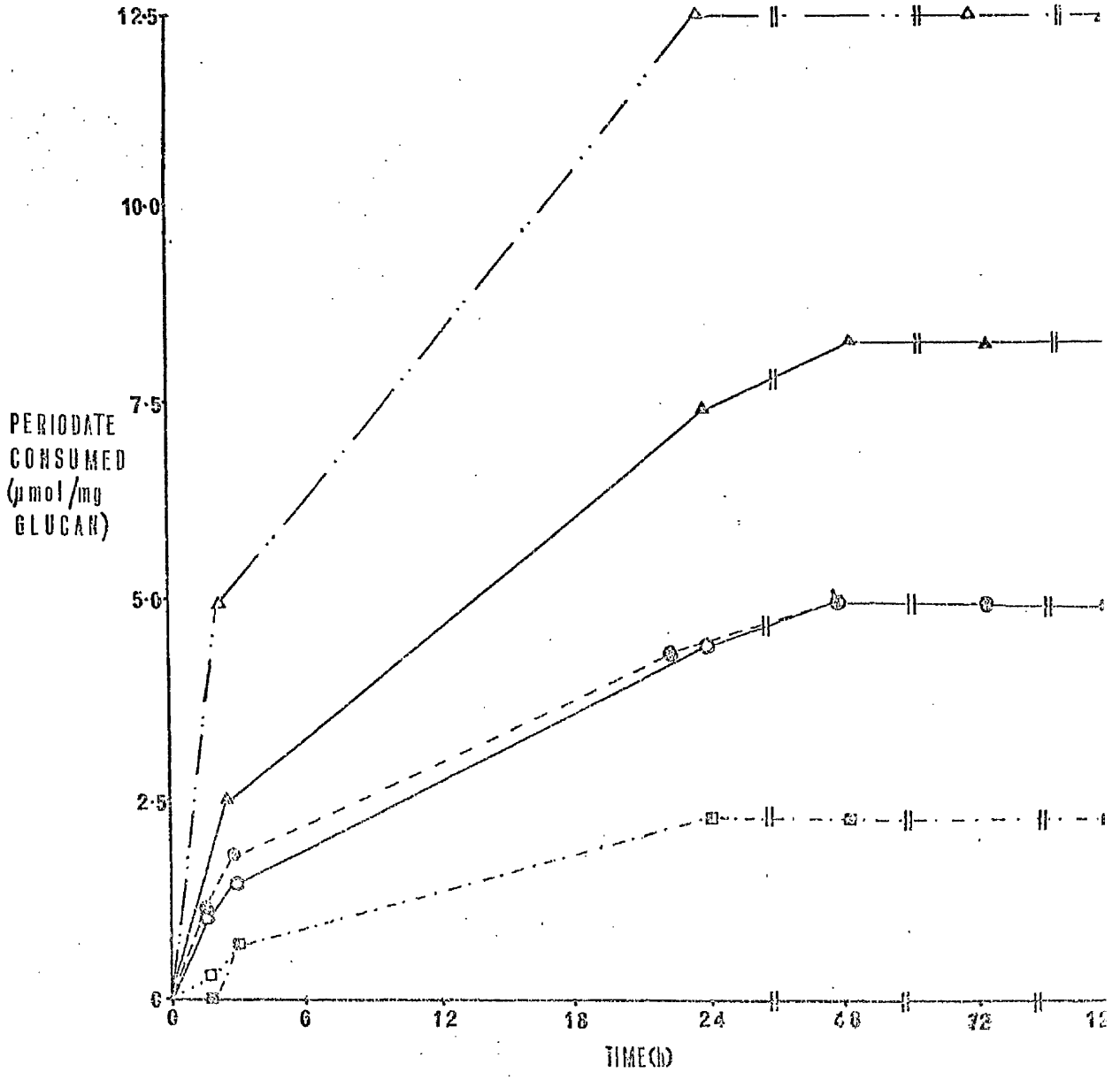


FIG.3.26 PERIODATE CONSUMPTION BY GLUCANS

The assay mixture contained 2mg/ml of dextran T2000 (△--- .. ---△), dextran I (△——△), dextran II (o - - o), dextran III (o——o), mutan 1 (□ ... □), or mutan 2 (■ - - ■) in 0·01M sodium meta-periodate solution in the dark at 4°C. Aliquots (3 ml) were removed at known time intervals and assayed for periodate content as in Section 2.15.8.

linkages, dextran I of 74%, dextrans II and III of 41% and mutans 1 and 2 of 18.9% '1 - 6 like' linkages. Allowance was made in the calculation for the degree of purity of the dextran preparation (Table 3.1) assuming that the contaminating material does not consume periodate.

3.5.4 Conclusions. The infra-red spectra suggest that dextrans I, II and III consist of α-glucopyranose ring units linked by α-(1→6)- and α-(1→3)- linkages but that the proportions of the linkages vary.

The results of the periodate oxidation confirm a difference in proportions of linkage types between dextran I and the other two dextran preparations, but suggest that dextrans II and III contain the same proportions of 'α-(1→6)-like' and α-(1→3)- linkages.

Oxidation of Dextrans I, II and III took a longer period of time to reach completion than the other dextran preparations and this could have been due to slower penetration of the dextran molecule by periodate due to the complex 'tree-like' structure of dextran. This could indicate that dextrans I, II and III have a more complex structure than dextran T2000. Dextran II was initially more rapidly oxidised than dextran III and this could indicate differences in steric arrangement of the molecules.

Enzymic degradation by α-amylase, mutanase and dextranase suggest that:-

(i) there are no α-(1→4)- linkages in dextrans I, II or III.

(ii) there are no α-(1→3)- linkages in dextrans II or III but that dextran I contains a small proportion of α-(1→3)- linkages.

(iii) all three dextran preparations contain α-(1→6)- linkages - dextran II having the highest proportion followed by dextran I and then dextran III.

However, the highly branched nature of dextran molecules could cause steric hindrance of the large enzyme molecule preventing its penetration to linkages shielded within the molecule. This could give a false impression about the

structure of a molecule - a highly branched one being less susceptible to enzymic breakdown than an unbranched one.

It can only be concluded, therefore, that dextran I is more susceptible to mutanase degradation than dextrans II and III and that dextran II is most susceptible to dextranase, followed by dextran I and then dextran III.

The purity and specificity of mutanase is also in question.

Mutanase has not yet been fully investigated and it readily hydrolyses amylopectin, glycogen and laminarin (Fig.3.24). The presence of contaminating enzymes and a lack of knowledge of the specificity of mutanase makes any interpretation of the results questionable.

However, the results of the structural studies on the three dextran preparations present evidence indicative of differences in their structure. These differences could be attributable to an increase in molecular weight of the dextran produced by successive enzyme samples, i.e. $\text{mol.wt.}_{I} < \text{mol.wt.}_{II} < \text{mol.wt.}_{III}$. Further studies would be necessary to ascertain the exact nature of these differences.

3.6. PARTIAL PURIFICATION OF DEXTRANSUCRASE

3.6.1 Hydroxylapatite chromatography. The dextransucrase preparation used was supernatant from peak II of Fig.3.12. It was investigated in order to see if more than one peak of enzyme activity could be found.

(i) Batch-wise elution. Batch-wise elution was not entirely satisfactory as the enzyme was not eluted sharply (Table 3.2). It was a lengthy procedure, working with large volumes of liquid and the recovery was low.

(ii) Column chromatography. The results of an experiment in which dextransucrase was adsorbed on to hydroxylapatite and then eluted with phosphate buffer after packing into a column are shown in Fig.3.27. Sodium phosphate buffer of the required

TABLE 3.2 BATCH-WISE ELUTION OF DEXTRANSUCRASE FROM HYDROXYLAPATITE.

Culture supernatant from phase II of a culture of S.sanguis grown at pH 7·1 ± 0·1 was eluted from 300 ml hydroxylapatite slurry by successively washing with the buffers listed as described in Section 2.12.1.i. The supernatant was assayed for dextransucrase as in Section 2.15.2ii and for protein as in Section 2.15.6i.

Molarity and pH of Sodium Phosphate Buffer.	Volume of Buffer used.	Specific Activity of Dextransucrase mg Dextran/mg Protein.
0·05 M, pH 6·0	250 ml	0·03
0·10 M, pH 6·0	250 ml	0·16
0·15 M, pH 6·0	250 ml	0·98
0·20 M, pH 6·0	250 ml	3·12
0·50 M, pH 6·8	250 ml	4·30

concentration crystallised out of solution at 4°C. It was necessary to change to potassium phosphate buffer which was soluble at all concentrations used at 4°C.

Elution from a column gave much more satisfactory results although dextransucrase was eluted over a fairly wide range of phosphate concentrations and the elution pattern was heterogeneous.

The assay of fractions for dextransucrase activity was carried out as in Section 2.15.2ii but the incubation period was reduced from 24h. to 4h due to the high activity of the samples. Samples with high enzyme activity formed a dextran gel within 4h. allowing them to be rapidly recognised and assayed.

The recovery was of the same order as batch elution (35-40%) but the specific activity of the eluate was much higher as elution continued and the final volume of liquid was smaller (Fig. 3.27). A 28-fold purification was obtained (Table 3.3).

3.6.2 Conclusions. Dextransucrase binds to hydroxylapatite from which it can be eluted with a linear potassium phosphate gradient. The heterogeneity of the elution pattern supports the view that there is more than one dextransucrase in the supernatant fluid.

3.7. PROPERTIES OF DEXTRANSUCRASE

3.7.1. Optimum pH. Dextransucrase activity was found to be optimal in the region 5·1 - 7·5 with a sharp drop in activity above pH 7·5 and a more gradual fall in activity below pH 5·1 to zero activity at 3·7 (Fig. 3.28). There was a scatter in enzyme activity in the region pH 5·1 - 7·5 which could not be attributed to the use of different buffers. The effect was reproducible for this enzyme sample.

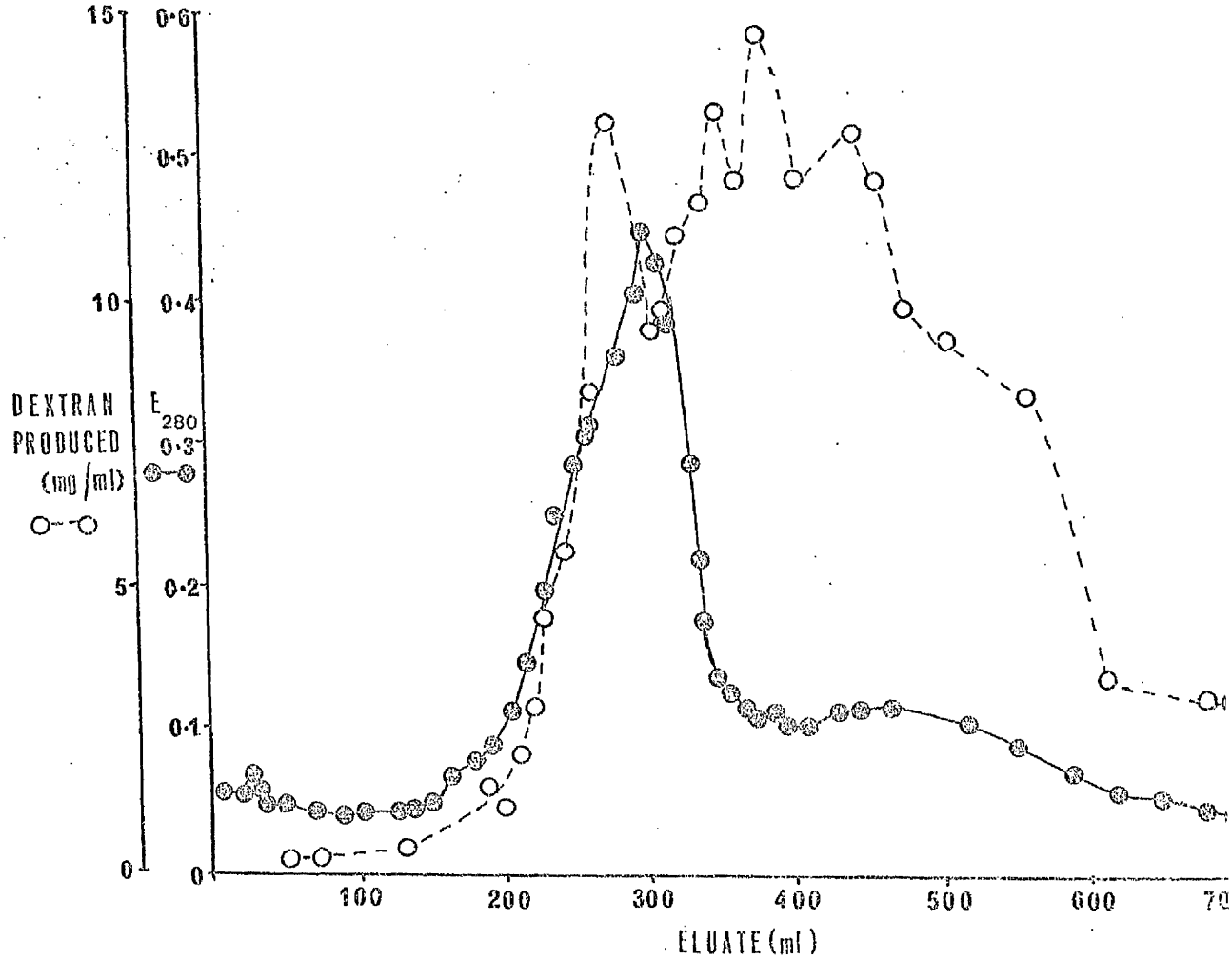


FIG. 3.27 HYDROXYLAPATITE CHROMATOGRAPHY OF DEXTRANSUCRASE II.

Culture supernatant from phase II of a culture of S. sanguis grown at pH 7·1 ± 0·1 was eluted from a column of hydroxylapatite with a linear gradient between 0·2M potassium phosphate buffer, pH 6·0 and 1·0M potassium phosphate buffer, pH 6·8 as described in Section 2.12.1ii. The U.V. extinction at 280 nm. (●——●) was measured. Samples (0·5ml) were assayed for dextransucrase as described in Section 2.15.2ii except that a 4h incubation period was used.

FIG.3.3 PURIFICATION OF DEXTRANSUCRASE II BY HYDROXYLAPATITE COLUMN CHROMATOGRAPHY (PRELIMINARY STUDY)

Procedure	Vol. (ml)	Enzyme Concentration (mg Dextran produced per ml).	Total Enzyme (mg Dextran) produced	Protein (mg/ml)	Specific Activity (mg Dextran produced/ ml).	Yield %	Purification
Original Culture Supernatant	1860	25·8	47,900	4·9	5 3	100	1
Washing Hydroxylapatite with 0·5M, 1·0M, 1·2M Potassium Phosphate Buffer pH 6·0.	450	58·2 #	26,200	/	-	55	-
Total enzyme eluted by column chromatography of hydroxylapatite	2020	4·8	9,650	0·06 ##	80	20	15
Pooled eluate samples 100-200 from column chromatography	360	7·5	2,700	0·05 ##	150	5·6 ^{s}	28 ^{s}

S Due to technical difficulties the purification procedures were delayed and enzyme activity was probably lost as a result.

* This figure was calculated from the total amount of enzyme in the original culture supernatant minus the amount of enzyme in the washings.

† Protein assay not possible due to faulty batch of antibiotics, leading to bacterial growth in samples.

Result is only an approximate estimate from U.V. absorbances due to growth of bacteria in samples.

It is stressed that the above work was a preliminary study only and would require to be repeated many times to achieve better results.

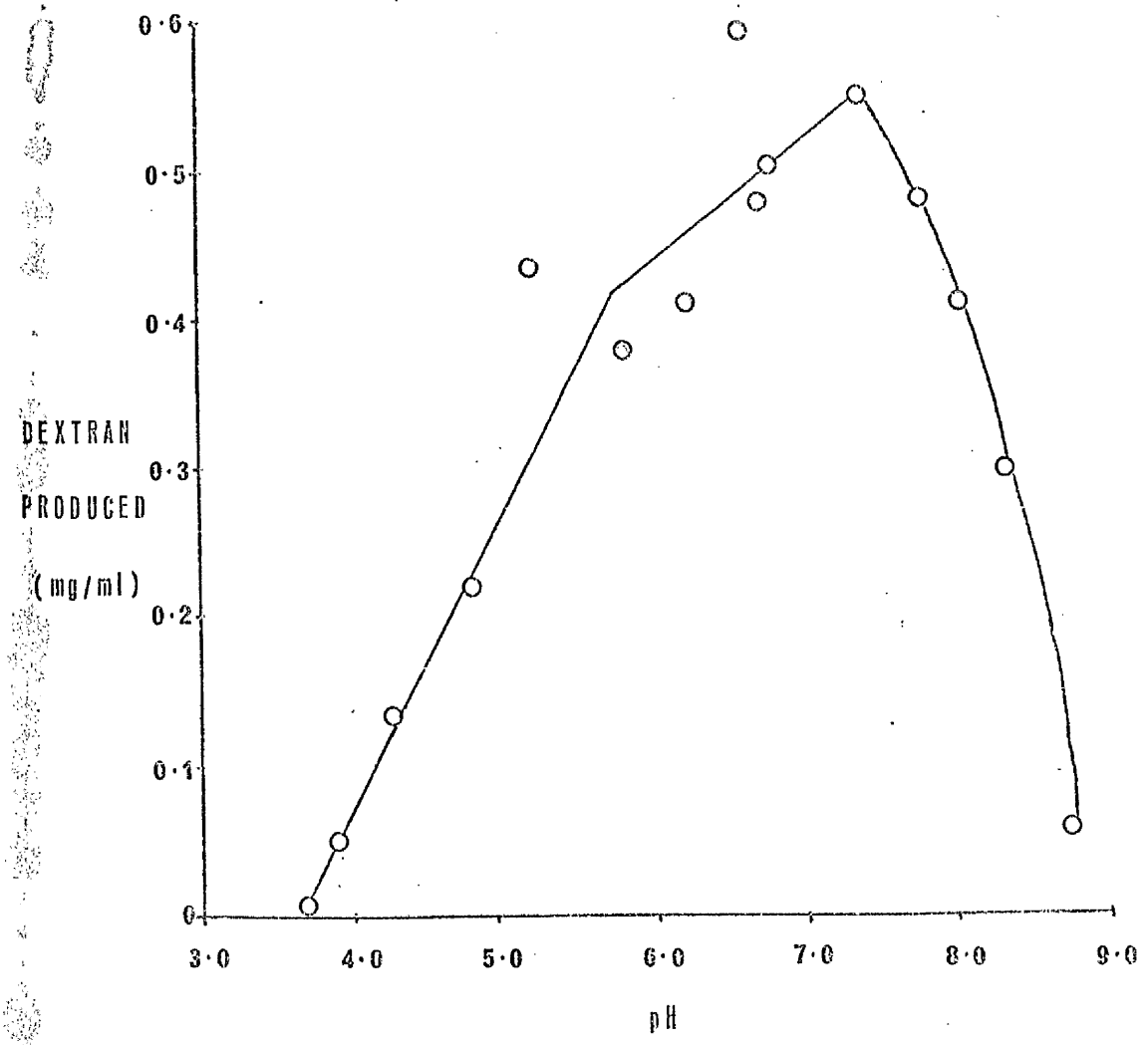


FIG.3.20 THE EFFECT OF pH ON DEXTRANSUCRASE ACTIVITY.

The assay mixture contained 0·5ml enzyme sample of phase II which had been partially purified by hydroxylapatite chromatography and 0·5ml buffered sucrose solution. The samples were assayed for dextransucrase as described in Section 2.15.2ii but the incubation period for all samples was reduced to 6h to simplify the procedure.

This scatter could be due to the presence of two or more enzymes of different specificities. The enzyme preparation from phase III had been partially purified by hydroxylapatite chromatography (Section 2.12.1i).

3.7.2. Stability.

(i) Storage. Crude supernatant samples could be maintained at $4^{o}C$ for an hour without any loss of activity, provided the pH was in the range 6 - 7·5. Storage at $4^{o}C$ for prolonged periods was accompanied by a fairly rapid loss of activity. Partially purified samples did not lose their activity so rapidly (Fig. 3.29).

Storage at $-20^{o}C$ was accompanied by a marked initial drop in activity (loss of 40%) within 3 days followed by a more gradual loss (to 5% of original activity) by 35 days.

(ii) Handling. Repeated freezing and thawing of enzyme samples caused a gradual drop in activity (35-60% activity lost after freezing and thawing four times). Fig. 3.30.

3.7.3. Conclusions. The pH optimum curve of the dextransucrase preparation used exhibited a 'scatter' of enzyme activity in the region pH 5·1 - 7·5. However, this enzyme preparation had been partially purified and a crude sample or one that had been harvested from another phase of enzyme production before purification might give a different pattern.

The reproducible scatter could be due to the presence of a particular combination of enzymes of different specificities. The enzyme or enzymes rapidly lose activity about pH 7·5 and below pH 5·5. This could explain the rapid loss of enzyme activity in cultures of S.sanguis without pH control (Fig.3.2) when the pH falls below 5·4. It also explains the low enzyme activity in cultures maintained at pH $8{\cdot}1 \pm 0{\cdot}1$.

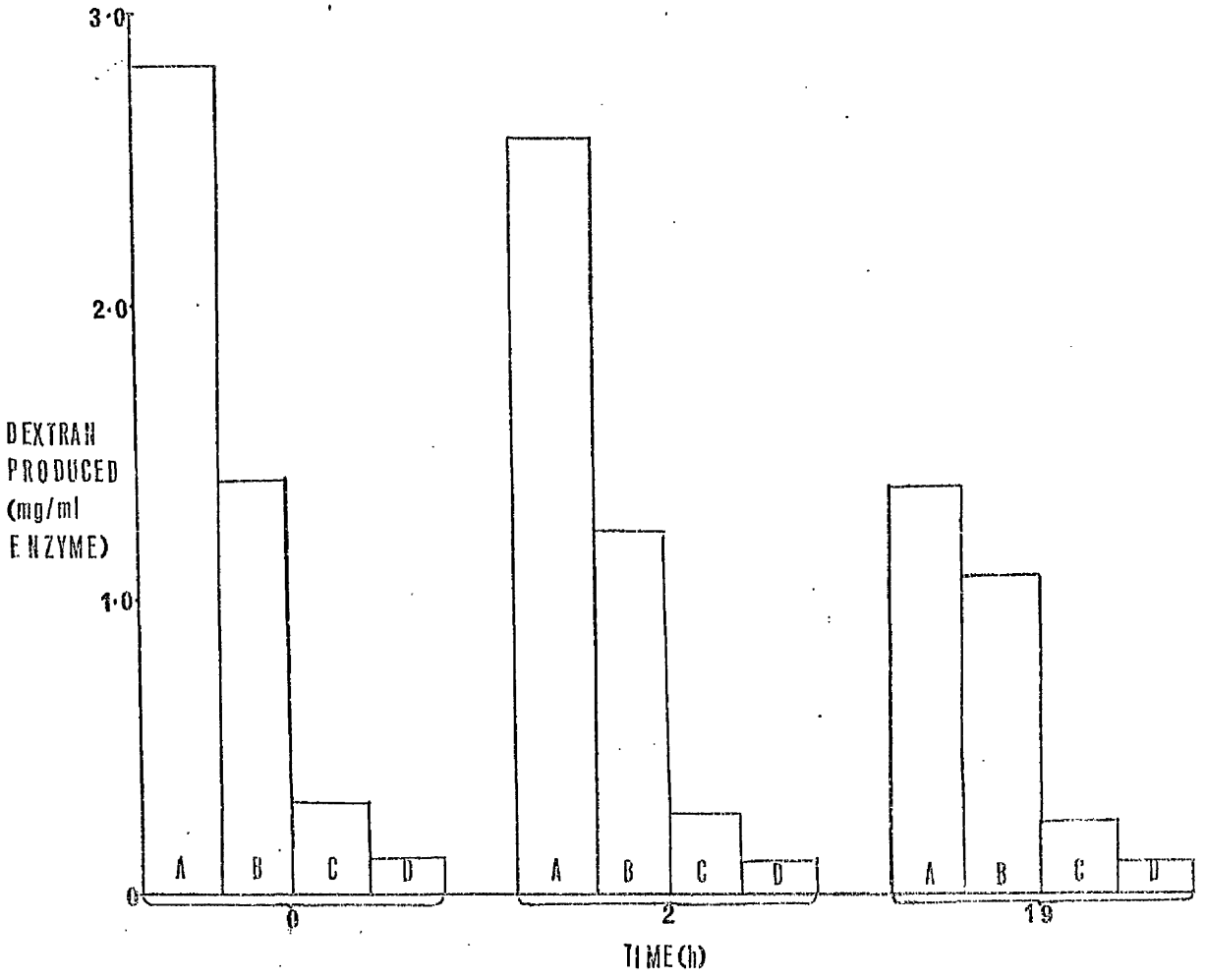


FIG.3.29 THE EFFECT OF STORAGE AT 4°C ON DEXTRANSUCRASE ACTIVITY.

An enzyme sample (A) which had been partially purified by hydroxylapatite chromatography (specific activity 9·2 mg/mg protein) was diluted 1 : 1 (B), 1 : 9 (C) and 1 : 19 (D) with 0·1M sodium phosphate buffer, pH 7·0 and assayed for dextransucrase activity as described in Section 2.15.2ii. They were then stored at 4°C for up to 19h and assayed at known time intervals.

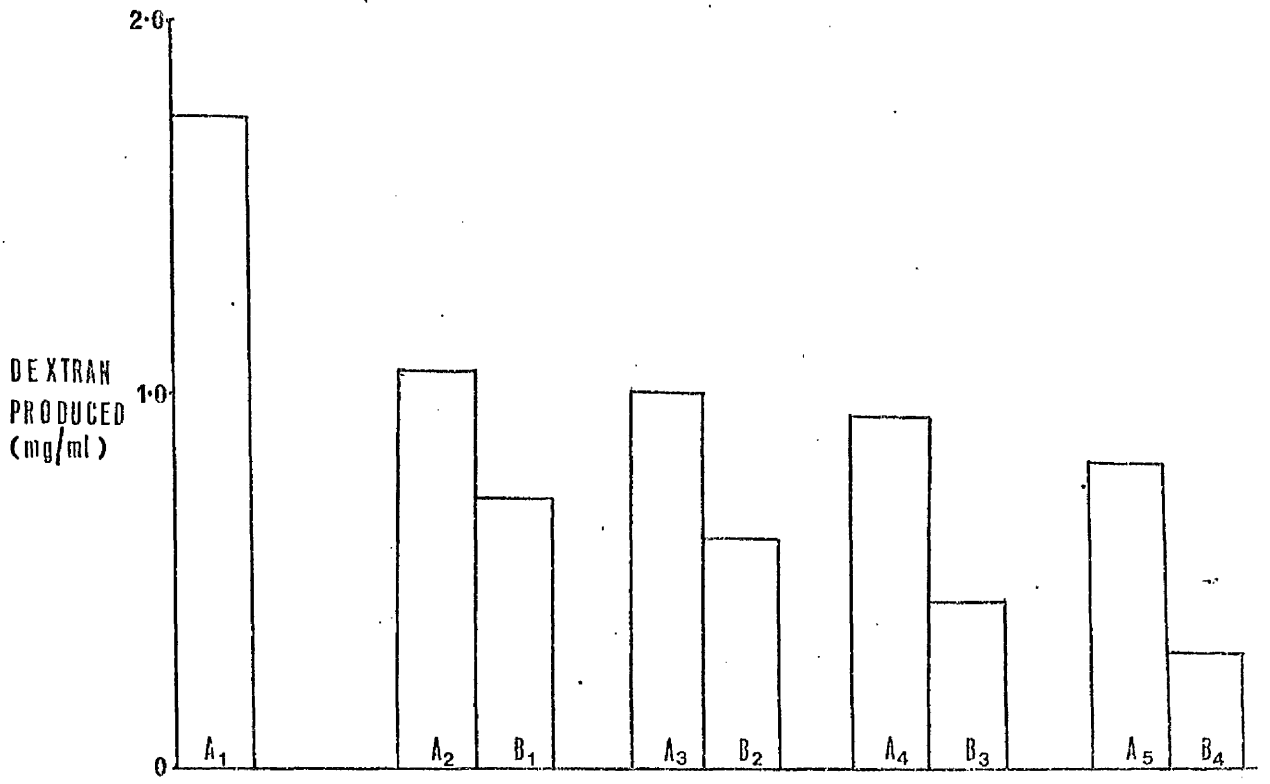


FIG.3.30 THE EFFECT OF FREEZING AND THAWING ON DEXTRANSUCRASE ACTIVITY.

A sample of crude supernatant from phase III of a culture grown at pH 7·1 ± 0·1 was assayed for dextransucrase activity as described in Section 2.15.2ii - (A_1), stored at -20°C for three days and thawed. It was again assayed for dextransucrase activity (A_2), frozen, immediately thawed and assayed again (A_3). This was repeated twice more (A_4 and A_5). A second enzyme sample from phase II of a culture grown at pH 7·1 ± 0·1 was similarly frozen and thawed four times in rapid succession and the dextransucrase content assayed at each stage (B_1, B_2, B_3 and B_4 respectively).

The enzyme is fairly stable for short periods at 4°C. and can be stored for longer periods at -20°C. However, the freezing and thawing of these samples causes loss of 8-15% of the enzyme activity each time.

4. Discussion and Conclusions.

S.sanguis 804 produces constitutive, extracellular dextransucrase when grown in Carlsson's diffusate medium in batch culture at 37°C with constant stirring. The organism is microaerophilic but grows rapidly under the above conditions, with the production of large amounts of acid, leading to a rapid drop in pH to values of approximately 5 and cessation of growth. However, work by Rosan and Eisenberg (1973) indicates that in the presence of even minute amounts of oxygen, S.sanguis produces hydrogen peroxide which builds up, leading to inhibition of growth (Donaghue, 1974) and this might be expected to occur under the above conditions. This, in turn, might lead to decreased dextransucrase production. It was not possible to examine the effect of the contaminant on growth of S.sanguis as the bacteria aggregated and there was insufficient time. However, Holmberg and Hallander (1973) have shown that addition of catalase to the culture medium prior to inoculation with S.sanguis did prevent the growth inhibition attributed to hydrogen peroxide. The effect of hydrogen peroxide build-up on dextransucrase production has not been investigated by these workers.

Growth and dextransucrase production by S.sanguis 804 ceased when the pH fell below 5.2 and the glucose in the medium had been utilised.

The cessation of growth may have been due to the build-up of hydrogen peroxide, as already discussed, or of some other noxious substance. Alternatively, it may be due to depletion of glucose from the medium, overcrowding of bacteria, an unfavourable pH or to a combination of these factors.

When growth ceases, dextransucrase levels decline only slowly at first (Fig.3.2). Thus the rate of loss of enzyme activity which may be due to degradation by proteases released into the medium, denaturation or instability of the enzyme at the low pH, is greater than the rate of enzyme production. Carlsson et al (1969) have shown that dextransucrase from S.sanguis 804 remains stable for only 1h at 37°C in the pH range 5.2 - 8.5 and that loss of activity is more rapid at lower pH values. It seems likely, therefore,

that the loss of enzyme activity is due to its instability at 37°C and particularly at low pH. Proteases released into the medium by the bacteria may also contribute to dextransucrase breakdown as might hydrogen peroxide accumulation. Maintenance of the pH at values above 5 and below 8.5 has been shown to give improved growth and dextransucrase yields with optimum dextransucrase production at pH 7·1 ± 0·1. *S. sanguis* will not grow below pH 5·0 which could explain the cessation of growth in the batch culture when the pH fell below 5·2. However, the gradual drop in pH from the initial 6·8 – 7·0 of the medium to 5·2 may have allowed the organism to acclimatise gradually to the low pH and this may not have been the only factor involved in preventing growth. Even at more favourable pH values growth ceased when all the glucose had been depleted (Fig. 3.12).

Dextransucrase production by *S. sanguis* 804 was greatly improved by the presence of a very small proportion of *Staph. aureas* or of a *Pseudomonas* species as contaminant. One possible but tentative explanation of this might be that the presence of catalase produced by the contaminant dispersed the hydrogen peroxide accumulating in the medium and allowed growth of *S. sanguis* to proceed.

At all pH values studied, a triphasic pattern of enzyme production was observed – the first small peak occurring as growth started, the second, larger peak when growth was maximal and the third and largest peak after growth ceased and all glucose had been utilised. The peaks were reproducible at all pH values studied and it therefore seemed unlikely to be due entirely to experimental error. Carlsson and Elander (1973) observed only one phase of enzyme activity when growing *S. sanguis* 804 in batch culture at pH 7·0. However, dextransucrase levels were not measured until growth of the organism was well into the logarithmic phase and the measurements were discontinued within three hours of depletion of the medium of the glucose. It is possible, therefore, that the first and last phases of enzyme production may have been missed.

These phases of enzyme production may be caused by fluctuations in the levels of inhibitors or stimulatory substances in the medium or by the presence of degradative enzymes such as proteases or dextranases. Other possibilities are the elaboration of a multi-enzyme system or the alteration of factors controlling the release of enzyme from the cell. However, Carlsson and Elander (1973) have been unable to demonstrate the presence of any inhibitors or inducers in S.sanguis 804 cultures grown under a variety of conditions and S.sanguis 804 does not produce dextranase (Walker, 1972). Variations in the release of the enzyme seems unlikely while the cells are still actively growing although an increase in enzyme at the autolytic phase of growth might be explained by the release of intracellular enzyme upon lysis of the cells. However, this would not be expected to make very much difference to the enzyme levels as only 10% of the dextransucrase produced by S.sanguis 804 is intracellular (Sharma et al, 1973) and lysis would probably occur gradually over a period of some hours. Lysis might be accompanied by the release of proteolytic enzymes which would probably have a more profound effect upon the dextransucrase by degrading it. Very little attention has been paid, in the literature, to the growth conditions required for optimal dextransucrase production or to the possibility of changes in the enzyme production pattern during growth. Of the various possibilities suggested, the most plausible explanation is that the triphasic pattern is indicative of a multi-enzyme system, the spectrum of enzymes changing as growth of the culture proceeds. Despite the conclusions of Senghoff and Mehra (1972) it seems unlikely that a single enzyme alone could be responsible for the synthesis of a molecule which contains at least two types of linkage. As it has been reported that streptococcal dextransucrase can usually be separated into three or more distinct, enzymically active proteins (Chludzinski et al, 1974, Fukui et al, 1974) and dextransucrase from S.sanguis 804 has been separated into three such components (Carlsson et al, 1969), it is possible that the three peaks of enzyme activity may correspond to the production of

more than one enzyme.

If this were the case and each enzyme performed a different function, e.g. one may form α-(1→6)- linkages, another α-(1→3)- linked branch points, etc. one would expect the structure of the dextrans produced at the three phases to differ. Further studies showed that this was indeed, the case. The alternative possible explanations for the elaboration of three phases, outlined above, would not be expected to cause differences in dextran structure but in amount of dextran only.

Examination of dextran produced from enzyme harvested at each phase of enzyme activity was, therefore, necessary to establish if any structural differences did exist.

It was first necessary to ensure that the preparations were, indeed, primarily dextran. The carbohydrate in all three preparations was found to be almost entirely a polymer of glucose when examined by gas-liquid chromatography and acidic hydrolysis. Examination of glucans of known structure in a parallel study ensured the validity of the results. It was necessary to dry all glucans to constant weight over P_2O_5 *in vacuo* to eliminate errors due to moisture content and these dried glucans were used in all subsequent structural studies. The dextrans all brought about rapid aggregation of washed cells of *S.mutans* OMZ 176 which is considered to be a highly specific identification procedure for dextran. Electron microscopic studies of aggregated cells revealed the presence of electron dense globules adhering to the cell surface. In some cases, branched chains of smaller globules could be seen and 'bridges' of electron dense material were apparent between cells. This material was not seen in the absence of dextran and the sizes of the globules and width of the globule chains agree with the sizes calculated by other workers for dextran molecules (Newbrun *et al*, 1971) and dextran protofibrils (Johnson *et al*, 1974) respectively. It would appear that the electron dense material is dextran which adheres to the surface of *S.mutans* cells and forms 'bridges' linking cells together. Johnson *et al*, (1974) observed both 'globular' and 'fibrillar'

dextrans produced by strains of S.mutans 655 but they believed that the fibrillar dextran was the more important form in respect of causing the cells to cohere and to adhere to the walls of vessels in which they grew. Periodate oxidation studies on the three dextrans revealed that Dextran I was 74% '(1→6)-like' whereas Dextrans II and III were both 41%. The oxidation of Dextrans I, II and III took longer than that of Dextran T2000, a straight chain α-(1→6)- linked glucan, which may be due to slower penetration of the structurally more complex streptococcal dextran molecules. The oxidation products were not identified as the primary aim of this part of the study was to determine differences in their reaction to periodate oxidation which might indicate differences in structure rather than to determine the precise structure of each dextran. Similarly, enzymic degradation studies using mutanase, amylase and dextranase were carried out in order to determine differences in susceptibility of the dextrans to different enzymes, which might be indicative of structural differences. Unfortunately, the mutanase preparation was not well characterised and appeared to be contaminated with other enzymes as it readily hydrolysed laminarin, amylopectin and glycogen as well as mutan. The specificity of the mutan-degrading activity is not known and this must cast some doubts on the relevance of its use. However, dextrans with the same structure would, under the same conditions, be degraded to similar extents by the mutanase preparation. Dextrans of different structures would be expected to differ in their susceptibility. The fact that Dextran I was relatively easily hydrolysed by mutanase and dextranase and oxidised by periodate suggests a relatively simple structure, allowing easy access of enzymes and periodate to susceptible linkages. Dextrans II and III were not hydrolysed by mutanase although the periodate oxidation results suggest a high α-(1→3)- linkage content. This probably indicates that the α-(1→3)- linkages are sterically inaccessible to the enzyme molecule. They may be shielded within the molecule by an 'outer layer' of α-(1→6) - linkages or the specificity of the mutanase may not

permit degradation of the α-(1→3) - linkages in the particular arrangement present in these molecules. Although Dextrans II and III contain similar proportions of '(1→6) - like' linkages, they are not degraded to the same extent by dextranase, Dextran II being much more susceptible to hydrolysis than Dextran III. This suggests that the α-(1→6) - linkages in Dextran II are more accessible than those of Dextran III and that structural differences exist between them.

The dextrans prepared from S. sanquis at stages I, II and III may consist of mixtures of dextrans. For example, dextran II may contain some dextran I and a small amount of dextran III but would be expected to be predominantly dextran II. Different proportions of each dextran in a mixture would, therefore, give different results for periodate oxidation and the time at which the enzyme was harvested for dextran production would be important. Carlsson et al (1969) harvested dextransucrase when all glucose had been utilised i.e., equivalent to phase II and the dextran prepared from this enzyme was found to be 52% '(1→6)-like' (Ceska et al, 1972). However, the enzyme was prepared at pH 6 when the pattern of enzyme production would be slightly different from that produced at pH 7·0 (Fig. 3.15) and different proportions of the dextrans could be expected. From Fig. 3.15, it appears that a higher proportion of dextransucrase I is produced at pH 6 than at pH 7 and one would expect a higher proportion of Dextran I and, therefore, a higher α-(1→6)- linkage content. This might explain why Carlsson et al (1969) obtained results indicative of a higher (1→6) - like linkage content than the present study. Alternatively, the dextran may change gradually through the growth period from one with a high α-(1→6) - linkage content to one with a higher α-(1→3) - linkage content in which case one would expect enzyme harvested at phase II to have an α-(1→6) - linkage content intermediate between those harvested at phase I or III. This would be supported by the values of 74% for Dextran I in this study, 52% for Dextran II

in Carlsson et al's (1969) study and 41% for Dextran III in this study but it does not explain why Dextrans II and III of this study appear to have the same α-(1→6) - linkage content as indicated by periodate oxidation. Possibly the transition from Dextran II to III is accompanied by an increase in molecular weight or complexity of structure which does not alter the proportions of the linkage types. The (1→6) - like linkage content (18·9%) of mutan prepared from S.mutans OMZ 176 was in fairly close agreement with that obtained by other workers (Guggenheim, 1970; Newbrun, 1972) using mutan prepared under similar conditions.

Infra-red spectroscopy supported the conclusion that the dextrans were all homopolymers of α-glucopyranose units with α-(1→6) - and α-(1→3) - linkages. The spectra for the three dextrans were all very similar but differences did exist, supporting the view that they do differ structurally. Basic similarities of overall pattern could be seen between the dextrans but were not apparent on comparison of i.r. spectra of dextran with those of other glucans (Fig. 3.22-3.23). All the glucans studied including purchased reference compounds were prepared and examined under the same conditions, as Heyn (1974) has demonstrated that the method of preparation can have a profound effect upon the results. For example, variations in moisture content of samples of the same dextran would result in spectra with the same pattern of peaks and bands but the trace would be 'shifted' in one direction or the other, depending on the amount of moisture contained in the molecule. Freeze-dried dextrans have characteristic peaks in the i.r. spectrum. Comparison of spectra produced from glucan samples prepared under different conditions, which is common in the literature, should not, therefore, be undertaken without due consideration to the method of preparation, as misleading interpretations might be made. The increase in the molecular weight of a dextran does not have a profound effect upon the i.r. spectrum but changes in the proportions of different linkages and hydrogen bonding do bring about changes in the

spectrum proportional to the degree of change in structure. Examination of the three dextran preparations revealed a gradual transition in their spectra from Dextran I through II to Dextran III, indicative of a gradual change in structure. The i.r. spectra for Dextran III and mutan 1 were almost identical.

It is difficult to draw definite conclusions about the structure of a particular dextran from its i.r. spectrum as the significance of many of the peaks and bands is unknown. A thorough study of dextrans produced by Leuconostoc species has been carried out by Heyn (1974) but this was reinforced by a considerable knowledge of the chemical properties and structure of a large number of these dextrans. This made it possible to reach various conclusions about the structure of 'new' dextrans by comparison of their spectra with those of dextrans of known structure. However, the structures of streptococcal dextrans are still unknown and very little information has been accumulated in respect of their chemical properties. When such information becomes available it may be possible to assign dextrans to structural groups.

The results of these structural studies suggest that Dextran I is a fairly simple molecule with a high α-(1→6) - linkage content (74%) and a higher proportion of α-(1→3) - linkages than mutan. This is supported by its lability to dextranase and mutanase. Dextran II appears from periodate oxidation studies to have a higher α-(1→3) - linkage content than Dextran I although it is not hydrolysed by mutanase and is more readily degraded by dextranase than is Dextran I. A possible explanation of this is that the increase in complexity of one molecule could lead to steric hindrance of the mutanase, preventing it from reaching susceptible linkages sheltered within the molecule. It would seem that α-(1→6) - linkages are more readily available to dextranase. Dextran III would appear from periodate oxidation studies to have the same linkage content as Dextran II and, like Dextran II,

it is not degraded by mutanase. Unlike Dextran II it is not highly susceptible to dextranase but is very similar in this respect to mutan. The i.r. spectra of Dextran III and mutan are also very similar.

Again, increases in molecular weight, degree of branching and perhaps the formation of cross-links might explain the differences between Dextrans II and III.

The formation of intramolecular and intermolecular hydrogen bonds between the hydroxyl groups of dextran molecules has been postulated for Leuconostoc dextrans by Heyn (1974) but such bonds may also exist in streptococcal dextrans. Hydrogen bonding of a stronger type between water molecules and two hydroxyl groups to form a 'bridge' may also occur and molecules may be bound together by such bonds. Evidence put forward by Johnson et al (1974) and Newbrun et al (1971) suggests that streptococcal dextrans may indeed form aggregates, short chains of molecules and fibres consisting of more than one dextran molecule.

Certain ions have been shown to be incorporated into dextrans (Rorem, 1955, Kelstrup and Funder-Nielson, 1972) and are extremely difficult to remove. Certain divalent ions, such as calcium, may form 'bridges' by combination with negatively-charged groups within the dextran. The latter are thought to be ions which are reversibly bound to dextran but the mechanism is not understood (Rorem, 1955, Kelstrup and Funder-Nielson, 1972, Rölla and Mathieson, 1970).

It is generally believed that as the α-(1→3) - linkage content of dextrans increases, so the solubility of that dextran decreases. One would, therefore, expect the dextran from phase I to be more soluble than that produced later in the growth of S.sanguis as the latter contain approximately 41% α-(1→6) - linkages.

If this were the case, it might be compared to that observed in dextransucrase

production by Leuc-mesenteroides (Smith, 1970-71), S.bovis (Gold et al, 1973) and S.mutans SL-1 (De Leon et al, 1974), when the production of a water-soluble dextran preceded the production of a water-insoluble dextran. Dextran I might, therefore, be a precursor to the production of Dextrans II and III. Fukui et al (1974) have also shown that S.mutans HS-6 produces at least two dextransucrases - one of which elaborates a 94% α-(1→6) - linked, soluble dextran and the other/s produce insoluble dextran. Work by Ceska et al (1972) on dextran produced by dextransucrase from S.sanguis 804 has shown that the greater the purification of the enzyme used to prepare the dextran, the higher the α-(1→3) - linkage content of the dextran. This is also consistent with the production of several dextransucrases and the selection of one of these enzymes above the others in the course of the purification. Thus the proportions of each enzyme would alter with each purification step and the resultant dextran would have a different structure. Preliminary attempts at purification of dextransucrase by hydroxlapatite chromatography produced most of the enzyme activity in a single peak with a small shoulder (Fig.3.27) but this was followed by a second peak over a fairly wide phosphate concentration. The results were poor in respect of yield and purification (Table 3.3) but this work was only a preliminary investigation and requires a great deal of development and repetition in order to obtain more satisfactory results. However, the results were similar to those obtained by Carlsson et al, (1969) using batch chromatography and they suggest the presence of more than one protein with enzymic activity. The peaks of enzymic activity overlap and it is possible that minor peaks are obscured. In pooling some of the fractions for subsequent purification procedures, such minor enzyme components may be lost. It could be very useful to employ equipment which maintains the phosphate concentration at the level at which a particular species with enzymic activity is eluted until all protein from a particular peak has been removed from

the column. In this way, each protein peak could be eluted separately, avoiding overlap and minor peaks would become apparent.

Attempts at purification of dextransucrase have tended to rely primarily on hydroxylapatite chromatography employing potassium phosphate buffers at pH 6·8 and isoelectric focussing. However, Bernardi et al (1972) have shown that the use of potassium phosphate buffers at pH 5·8 or 7·8 instead of pH 6·8 for hydroxylapatite chromatography could advance or delay elution of proteins from the column and thus improve separation. They have also shown that the use of NaCl or $CsCl_2$ gradients can successfully be used to elute certain proteins from hydroxylapatite. Use of these refinements might improve the purification of dextransucrase. As already mentioned, the use of apparatus to maintain the phosphate level at that required by a particular protein component until elution was complete would help to separate the protein peaks and prevent obscuring minor peaks.

Carlsson et al (1969) have shown that isoelectric focussing of enzyme which had been harvested from S. sanguis 804 at the stage equivalent to phase III in the growth cycle revealed three protein components with enzymic activity. It would be interesting to examine the pattern of enzyme components, as revealed by isoelectric focussing, from samples of partially purified enzyme prepared from each of the three phases of enzyme activity. In this way, it might be possible to observe changes in the pattern of enzymic components as the growth of S. sanguis proceeds. Similarly, it might be possible to compare certain properties, such as pH optimum and molecular weight characteristics of the three enzyme preparations. The latter might be carried out by ultracentrifugation of the sample in a density gradient of, for example, glycerol or $CsCl_2$. Evidence that S. sanguis 804 dextransucrases can be dissociated into six protein components by sodium dodecyl sulphate (SDS) chromatography albeit with loss of enzymic activity, suggests another possibility (Dürt et al, 1974) namely dissociation of the enzyme into

subunits. Complete dissociation of the enzyme is accompanied by loss of enzyme activity but it seems possible that partial dissociation of the enzyme might occur in which one of the resultant fractions might retain enzymic activity. There might be several different permutations of the protein subunits which could retain activity and the partial dissociation of the enzyme might give rise to several molecules of different sizes with enzymic activity and with different specific activities. This could result in the production of several different enzymically active components with different isoelectric points and the loss of a large amount of the protein material as the inactive subunits of the molecules. Presumably some of the enzyme would also be completely dissociated causing a loss of a proportion of the activity and a low enzyme yield. This might explain why so many different enzyme fractions have been obtained from streptococcal dextransucrase (Guggenheim and Newbrun, 1969, Osborne et al, 1973), why it is sometimes necessary to load a large amount of the protein onto an electrophoresis column to obtain even small bands of active protein (Newbrun, 1971). Fukui et al (1974) have shown that one dextransucrase, produced from S. mutans HS-6 does not dissociate into subunits during SDS gel electrophoresis. This enzyme is relatively small (mol. wt. 70,000) and probably consists of only one protein molecule.

S. sanguis 804 dextransucrase forms a precipitate at the bottom of the column during isoelectric focussing in a liquid density column but this precipitation is prevented by the addition of urea or glycine. Further to this tentative suggestion, Dart et al (1974) have shown that they could not restore the activity of the completely dissociated enzyme by recombining the six fractions but they did observe that pretreatment of the enzyme with urea prior to SDS electrophoresis resulted in the production of an additional larger protein.

The pH optimum of dextransucrase prepared from S.sanguis was found to be 5·2 – 7·5 with a shoulder at approximately pH 5 – 6 and a peak at 7·0 – 7·5. This agrees with results obtained by Carlsson et al (1969), although they did not obtain a pronounced shoulder and peak in their pH optimum graph. However, other workers obtained results which are very similar to those obtained in this study. These workers, studying dextransucrases of streptococcal origin, also found that they exhibited wide pH optima with a shoulder in the region 5 – 6 and a peak at 7·0 – 7·5 (Gibbons and Nygaard, 1968, Balliet and Chang, 1974). Guggenheim and Newbrun (1969) have shown the dextransucrase of S.mutans HS with pH optimum 5·0 – 7·5 can be separated into several different dextransucrases with different pH optima, which collectively cover the range 5·0 – 7·5.

It would be interesting to examine and compare the pH profiles of enzyme harvested at phases I, II and III to see if they differ. However, a preliminary examination only of the pH profile of S.sanguis dextransucrase was possible due to shortage of time and for this, dextransucrase II was used. Carlsson et al (1969) used dextransucrase II which had been prepared at pH 6 and partially purified by hydroxylapatite chromatography and isoelectro focussing.

The results of the work carried out on dextransucrase from S.sanguis 804 and that obtained by other workers from streptococcal dextransucrase suggests the elaboration of several different enzymically active proteins, some of which may represent the dissocation products of other enzymically active proteins. The triphasic pattern of dextransucrase production by S.sanguis 804 may represent the elaboration of more than one dextransucrase and, concurrent with this, there are changes in the structure of the dextrans produced during growth in batch culture.

It is clear that the complexity of the system involved will necessitate a considerable amount of further research before the mechanism of action of dextransucrase and hence the role of sucrose in plaque formation and caries will be fully appreciated.

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